INVESTIGATING THE IMMUNE SYSTEM IN CHRONIC KIDNEY DISEASE – THE SONIC STUDY.

By

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ABSTRACT

Chronic kidney disease (CKD) is associated with a high infective burden and poorer vaccine responses. This thesis presents findings of a prospective observational study, where a non-biased approach was taken to systematically characterise the immune "landscape" in CKD and examine adaptive immune cell phenotypes associated with responses to two vaccines - seasonal trivalent influenza (TIV) and 23-valent pneumococcal polysaccharide (PPV23) - in older adults with and without CKD.

Despite significantly higher rates of self-reported infections, reductions in humoral responses to vaccination in patients with CKD were subtle, but with evidence of hyporesponsiveness to repeat PPV23 vaccination. CKD patients had fewer circulating T and B lymphocytes than controls, greater proportions of "Th2-like" ($CCR4^{+}CCR6^{-}CXCR3^{-}$) and T_{reg} cells ($CD4^{+}CD25^{+/high}FoxP3^{+}$) and fewer B_{regs} ($CD19^{+}CD24^{high}CD38^{high}$), but similar naïve/memory populations and "senescence"-associated T cells (defined by loss of surface CD27/28 and/or gain of CD57/KLRG1). Unexpectedly, CMV was the main determinant of T cell phenotype in both study groups and was also associated with poorer responses to both vaccines. Examination of neutrophil function demonstrated reduced oxidative burst capacity, reduced NETs generation and impaired migratory accuracy to fMLP in patients with CKD.

This study highlights the complexity of CKD-associated immune dysfunction and the important role played by CMV.

For my daughter Suzy,

who was there at the beginning

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ABBREVIATIONS

A&E	Accident & Emergency department
AAAAI	The American Academy of Allergy, Asthma and Immunology
Ab	Antibody
ACR	Albumin/creatinine ratio
ADMA	Asymmetric dimethylarginine
AF	Atrial fibrillation
Ag	Antigen
AGE	Advanced glycation end product
AID	Activation-induced cytidine deaminase
AKI	Acute kidney injury
AMR	Antibody maintenance ratio (expressed as geometric mean)
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC (fluorochrome)	Allophycocyanin
APC-Cy7	Allophycocyanin conjugated to cyanine 7

ARIC	Atherosclerosis Risk in Communities study
ARR	Antibody response ratio (expressed as geometric mean)
ASC	Antibody secreting cell
ATCC	American Type Culture Collection
BAFF	B cell activating factor
BCR	B cell receptor
BM	Bone marrow
BMI	Body mass index
BP	Blood pressure
BPH	Benign prostatic hypertrophy
BPPV	Benign paroxysmal positional vertigo
B_{reg}	Regulatory B cell
BSA	Bovine serum albumin; Body surface area
BV e.g. BV421	Brilliant violet fluorochrome
C1INH	C1 inhibitor
C4BP	C4b-binding protein
CA	California (US state)

Ca	Calcium
Ca-P	Calcium-phosphate product
CAP	Community acquired pneumonia
CCF	Congestive cardiac failure
CCI	Charlson Comorbidity Index
CCR	Chemokine receptor family
CD	Cluster of differentiation
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CR1	Complement receptor 1
CRP	C-reactive protein
CSR	Class switch recombination
CTLA	Cytotoxic T lymphocyte antigen

CVA	Cerebrovascular accident (stroke)
CVD	Cardiovascular disease
CXCL8	Chemokine ligand
CXCR	Chemokine receptor family
DAF	Complement-decay accelerating factor
DALY	Disability-adjusted life year
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DHR-123	Dihydrorhodamine 123
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DT	Diphtheria toxoid
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate

ELISA	Enzyme-linked immunosorbent assay
ENT	Ear, nose and throat
EPO	Erythropoietin
ERCP	Endoscopic retrograde cholangio-pancreatography
ESA	Erythropoiesis-stimulating agent
ESRD	End stage renal disease
FC	Fold change
FCS	Fetal calf serum
FGF23	Fibroblast growth factor 23
FITC	Fluorescein isothiocyanate
fMLP	N-formylmethyonine-leucyl-phenylalanine
FMO	Fluorescence-minus-one
FO	Follicular (B cells)
FoxP3	Forkhead box P3
FPR	Formyl peptide receptor
FPRL1	Formyl peptide receptor-like 1
FSC	Forward scatter

GALT	Gut-associated lymphoid tissue
GC	Germinal centre
GFR	Glomerular filtration rate
GI	Gastrointestinal
GMC	Geometric mean concentration
GMT	Geometric mean titre
GP	General practitioner
H2RA	Histamine-2 receptor antagonist
HA	Haemagglutinin
HAI	Haemagglutination inhibition
Hb	Haemoglobin
HbA1c	Glycated haemoglobin
HBsAg	Surface antigen of hepatitis B virus
HBV	Hepatitis B virus
HEF	Haemagglutinin-esterase-fusion protein
HI	Heat inactivated
Hib	Haemophilus influenzae B

HIV	Human immunodeficiency virus
HRA	Health Research Authority
hsCRP	Highly sensitive C-reactive protein
HSV	Herpes simplex virus
HTN	Hypertension
IFN	Interferon
Ig	Immunoglobulin
IHD	Ischaemic heart disease
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
IPD	Invasive pneumococcal disease
IQR	Interquartile range
IV	Intravenous
K^+	Potassium
KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LAV	Live attenuated vaccine

LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
MA	Massachusets (US state)
MAC	Membrane attack complex
MACS	Magnetic-activated cell sorting
MALT	Mucosal-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MASP1	Mannan-binding lectin serine protease 1
MBL	Mannose-binding lectin
MBL	Monoclonal B-cell lymphocytosis
MCP	Membrane cofactor protein
MCV	Mean cell volume
MD	Maryland (US State)
MDRD	Modification of Diet in Renal Disease study
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MZ	Marginal zone of spleen

NA	Neuraminidase
Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural killer cell activating receptor
NET	Neutrophil extracellular trap
NFκB	Nuclear factor κB
NHS	National health service
NIBSC	National Institute for Biological Standards and Control
NK cell	Natural killer cell
NKG2D	Natural killer group 2D
NLR	Neutrophil/lymphocyte ratio
NLRP3	Nucleotide-binding domain leucine-rich repeat family, pyrin domain containing 3
NOAC	New oral anticoagulant
NOD	Nucleotide-binding and oligomerisation domain
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
P	Phosphate

PAF	Paroxysmal atrial fibrillation
PAMP	Pathogen associated molecular pattern
PB/PC	Plasma blasts/cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PE	Phycoerythrin
PE-Cy7	Phycoerythrin conjugated to cyanine 7
PerCP-Cy5.5	Peridinin chlorophyll-A protein conjugated to cyanine 5.5
PI	Phagocytic index
PIS	Patient information sheet
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
Pn	Pneumococcus (*Streptococcus pneumoniae*)
PNG	Polymorphonuclear granulocyte
PnPS	Pneumococcal polysaccharide

PPI	Proton pump inhibitor
PPM	Permanent pacemaker
PPV23	23-valent pneumococcal polysaccharide vaccine
PRR	Pathogen recognition receptor
PTH	Parathyroid hormone
RAAS	Renin-angiotensin-aldosterone system
RAGE	Receptor for advanced glycation end products
RDE	Receptor destroying enzyme
rhEPO	Recombinant human erythropoietin
RNA	Ribonucleic acid
RORγt	Retinoid-related orphan receptor γ t
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RPMI-PS	RPMI-1640 medium supplemented with 1% penicillin/streptomycin
RRT	Renal replacement therapy
RT	Room temperature
SAD	Selective antibody deficiency

SAE	Significant adverse event
SASP	Senescence-associated secretory phenotype
SCr	Serum creatinine
siRNA	Small interfering ribonucleic acid
SONIC	Investigating the immune System in chrONIC kidney disease study
SSC	Side scatter
ssRNA	Single strand ribonucleic acid
Tbet	Truncated Brunauer-Emmett-Teller
T_{CM}	Central memory T cell
TCR	T cell receptor
TD	T cell dependent
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memoty T cell re-expressing CD45RA
Tfh	Follicular T helper cell
TGF	Transforming growth factor
Th	T helper cell
TI	T cell independent

TIA	Transient ischaemic attack
TIBC	Total iron binding capacity
TIV	Trivalent inactivated influenza vaccine
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
TT	Tetanus toxoid
TURP	Trans-urethral resection of the prostate
UHBFT	University Hospitals Birmingham NHS Foundation Trust
UK	United Kingdom
URTI	Upper respiratory tract infection
USA	United States of America
USS	Ultrasound scan
UTI	Urinary tract infection
V, D, J gene segments	Variable, Diversity and Joining gene segments
VA	Virginia (US State)

VDR	Vitamin D receptor
WCC	White cell count
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 The immune system in humans – an overview

All mammals, including humans, exist in close association with microorganisms. Although the "rules of engagement" between host and microbe remain undefined, it is clear that microorganisms can have either a beneficial symbiotic relationship (as seen with bacterial colonisation of the intestine) or a damaging effect on the host, in which case these microorganisms are referred to as pathogens. Detrimental effects of host-microbe interaction have led to the evolution of a number of immune defence mechanisms to protect the host (1, 2).

Three major immune defence mechanisms exist in humans: anatomical and physiological barriers e.g. skin and mucociliary clearance in the respiratory tract, together with innate and adaptive (also referred to as acquired) immune responses (3). A key feature distinguishing innate from adaptive immunity is the mechanism of pathogen recognition. Innate immunity relies on pattern recognition receptors (PRRs) – a limited repertoire of germline-encoded receptors with broad specificities, recognising highly conserved microbial structures or products of infection (1). This allows rapid (within hours) mobilisation of immune effector responses against pathogens. In contrast, the adaptive immune system relies on an extremely diverse repertoire of antigen receptors, but with narrow specificities, which arise from essentially random somatic recombination of germline gene segments (3). The trade-offs for the ability to recognise virtually any antigen are a time delay in generation of effective adaptive immune responses (approximately 5 days is required for clonal expansion of effector cells to clear pathogens) and the risk of inappropriate immune activation to self-antigens, or autoimmunity, which has led to the evolution of a number of tolerance mechanisms (1).

Another important distinction between innate and adaptive immune systems is the ability of adaptive immunity to generate antigen-specific immunological memory, with augmented effector responses upon pathogen re-encounter (3). This mechanism is the basis for vaccination as a therapy to reduce burden of infectious disease - discussed later in this chapter.

1.2 The innate immune system

The innate immune system has both cellular and humoral components, which are summarised in Table 1-1. Description of the function of all innate immune components is outside the scope of this Chapter, but the main features pertinent to pathogen clearance are described in the following sections.

1.2.1 Innate immune system pathogen recognition mechanisms

Three broad strategies for pathogen recognition are employed by the innate immune system (summarised in Table 1-2): the detection of "microbial non-self" or pathogen-associated molecular patterns (PAMPs), detection of "danger" signals or damage-associated molecular patterns (DAMPs) associated with infection and inflammation, and the detection of "missing self" – molecules present on healthy cells that are downregulated with infection (2).

Table 1-1 Summary of innate immune system components and their main functions.

GI: gastrointestinal; NET: neutrophil extracellular trap; CRP: C-reactive protein; NK cell: natural killer cell. Compiled from references (2-4).

Component	Main function	Location
Non-haematopoietic cells		
Epithelial cells	Barrier – physical and physiological e.g. secretion of mucin, low pH environment of stomach; Chemokine and cytokine secretion;	Skin, airways, GI tract
Haematopoietic cells (myeloid lineage)		
Monocytes	Precursors for tissue-dwelling macrophages; Phagocytosis (poor); Cytokine & chemokine production;	Blood
Macrophages	Phagocytosis Cytokine & chemokine production	Tissue resident e.g. Kuppfer cells (liver)
Neutrophils	Phagocytosis; NETs generation; Cytokine production.	Blood & tissues; migrate to site of injury in response to chemotactic signals
Dendritic cells	Antigen presentation to T cells Cytokine and chemokine production.	Tissue; migrate via blood & lymph to secondary lymphoid organs.
Eosinophils	Helminth defense – release of cytotoxic enzymes & proteins	Blood & tissues; migrate to site of injury in response to chemotactic signals.
Mast cells	Allergy Release of histamine, serotonin → vasodilation; Leucotrienes, prostaglandins →increased vascular permeability	Mucosal and connective tissues
Basophils	Allergy Anti-tumour responses	Blood
NK cells	Intracellular pathogen defense Direct cell lysis (perforin/granzymes) Cytokine production (Type 1 interferons)	Blood
Humoral components		
Complement	Opsonisation Recruitment of other immune cell types Direct cell lysis	Blood
Acute phase proteins e.g. CRP, pentraxins	Opsonisation	Synthesised in liver

Table 1-2 Summary of innate immune system pathogen recognition receptors.

PAMPs: pathogen associated molecular patterns; TLR: Toll-like receptor; NOD: nucleotide-binding and oligomerisation domain; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; DAMPs: damage-associated molecular patterns; NLRP3: nucleotide-binding domain leucine-rich repeat family, pyrin domain containing 3; RAGE: receptor for advanced glycation end products; MHC – major histocompatibility complex; KIR – killer cell immunoglobulin-like receptor. Adapted from reference (2) under the Creative Commons License.

	Receptor types
Detecting microbial "non-self" (PAMPs)	Transmembrane: • TLR • C-type lectin (e.g. dectin 1 fungal PAMPs) Intracellular: • NOD-like receptors (e.g. NOD1/2 - bacterial peptidoglycans) • RIG-1, MDA5 (viral RNA) • DAI (viral DNA)
Detecting common metabolic consequences of cell injury/infection (DAMPs)	• NOD-like receptors (e.g. in NLRP3 inflammasome activation) • RAGE
Detecting "missing self"	• MHC class 1-specific inhibitory receptors (e.g. KIR on natural killer cells)

The best characterised class of PRRs is Toll-like receptors (TLRs) – a group of dimeric transmembrane proteins that recognise conserved microbial components, which can be located both on the cell surface membrane or localised intracellularly (e.g. on endosomes) (3). The TLR family consists of 10 different receptors (summarised in Table 1-3), each of which recognises a limited and different repertoire of "microbial non-self" PAMPs (3, 5). Intracellular signalling activated by TLR ligation leads to activation of transcription factors including nuclear factor κB (NFκB) and generates an inflammatory response.

TLR-ligand interactions stimulate tissue-resident macrophages to secrete cytokines including tumour necrosis factor α (TNFα) and interleukins (IL) 1β and IL-6, which orchestrate local and systemic inflammation. Endothelial actions of TNFα and IL-1β induce vasodilation and increased vascular permeability, facilitating immune cell recruitment to the site of infection. Hepatocyte stimulation by IL-1β and IL-6 results in the production of various acute-phase proteins, including pentraxins and collectins, which opsonise microbes for phagocytosis by macrophages and neutrophils and also activate complement.

Table 1-3 Toll-like receptors (TLRs) and their known ligands.

Exogenous ligands denoted by black text, endogenous ligands highlighted in blue. dsDNA: double-stranded deoxyribonucleic acid; siRNA: small interfering ribonucleic acid; ssRNA: single strand ribonucleic acid. Adapted from reference (3) under the Creative Commons Licence.

TLR	Ligand(s)
TLR1	Lipopeptides, peptidoglycans
TLR2	Lipopeptides, lipotechoic acid, glycolipids
TLR3	dsDNA, siRNA mRNA
TLR4	Lipopolysaccharide, phosphorylcholine Heat shock protein, defensin 2, fibrinogen
TLR5	Flagellin
TLR6	Lipopeptides
TLR7 / 8	ssRNA, resiquimod Autoantigen-containing immune complexes
TLR9	CpG DNA Chromatin complex
TLR10	unknown

It is important to note that pathogen sensing occurs in both haematopoietic and non-haematopoietic cells (e.g. endothelial cells) and the magnitude of the elicited inflammatory response (and the potential to cause tissue damage) can be determined by "where" the pathogen is sensed. For example, sensing of widely disseminated blood-borne pathogens stimulates a robust systemic inflammatory response, contrasted by only localised inflammation in response to pathogen sensing at an immunological barrier e.g. skin (6).

1.2.2 Cellular effector mechanisms of the innate immune system

The main innate immune cellular effector mechanism is phagocytosis – the recognition, engagement, engulfing and subsequent destruction of pathogens - which can be performed by monocytes, macrophages, neutrophils and eosinophils – collectively termed "phagocytes" (4). Phagocytosis is essential for host defence against intra and extracellular bacteria, together with fungal pathogens. Innate immune cells also play a key role in co-ordinating the adaptive immune response through antigen presentation, which will be discussed later.

1.2.2.1 Phagocytic function of innate immune cells

Phagocytes engulf pathogens by creating a membrane bound vesicle (phagosome) with projections of their cytoplasmic membrane (pseudopodia), which then fuses with cytoplasmic granules containing "killing machinery", thus forming the phagolysosome. Two main killing mechanisms are employed: respiratory burst – an NADPH oxidase dependent process that yields the production of reactive oxygen species including hydrogen peroxide, and the action of non-oxygen-dependent toxic enzymes and cationic

proteins e.g. lysozyme and myeloperoxidase (7). Phagocytosis is facilitated by "marking" or opsonisation of microbes with products of the acute phase response, complement proteins and specific antibody. In addition to phagocytosis, neutrophils extrude intracellular DNA to form neutrophil extracellular traps (NETs), the function of which remains to be fully elucidated, but includes pathogen capture and thrombosis (8, 9).

1.2.2.2 Humoral effector mechanisms of the innate immune system

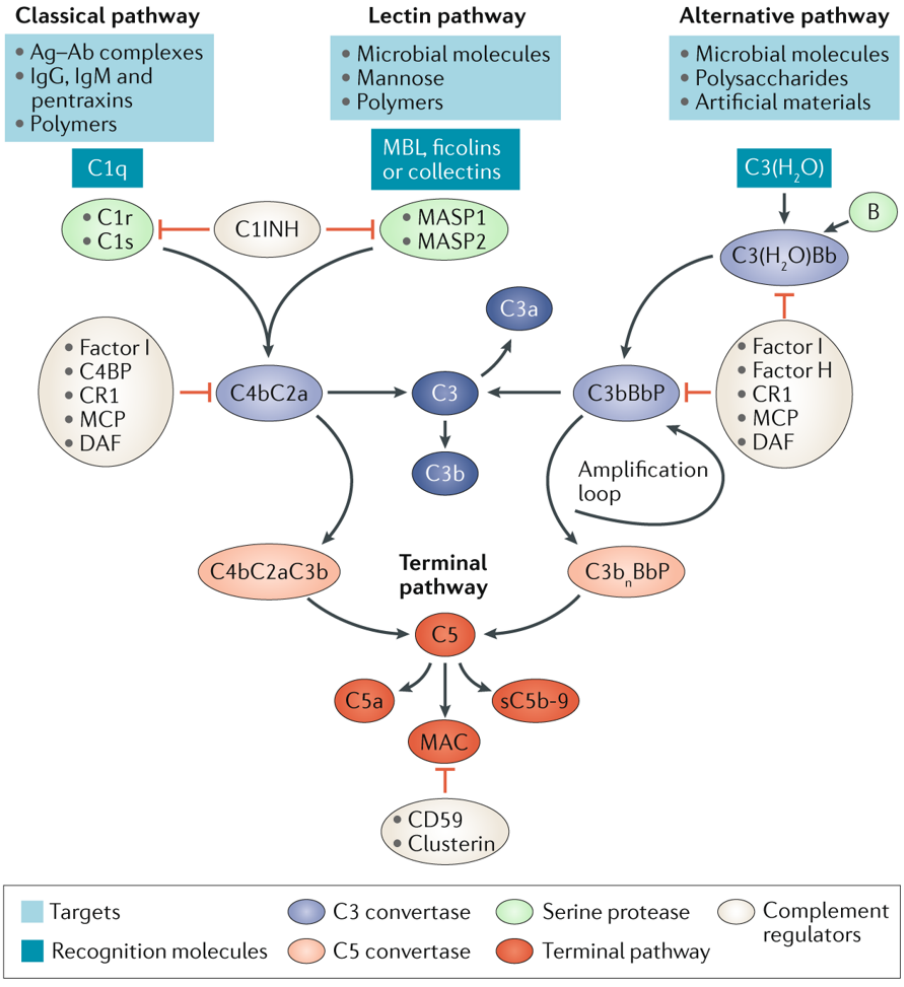
The main humoral component of the innate immune system is the complement system – a group of glycoproteins found in tissues and the circulation, the sequential activation of which is responsible for its 3 main functions: opsonisation, chemotactic activity and recruitment of phagocytes, together with direct cell lysis through formation of the membrane attack complex (MAC) (10). Figure 1-1 summarises the 3 pathways of complement activation and the main functions of individual components of the complement cascade.

1.3 The adaptive immune system

The hallmarks of adaptive immunity are immunologic specificity (with adaptive immune cells having an extremely diverse repertoire of antigen receptors with narrow specificities), discrimination of self and non-self (with the ability to remain inactive against self-antigens) and the ability to generate antigen-specific memory, with augmented effector responses upon antigen re-encounter (3).

Figure 1-1 Summary of complement cascade and pathways of activation

Complement activation can occur through classical, lectin and alternative pathways, initiating the assembly of C3 convertases (C4bC2a, C3bBbp), which cleave C3 to generate C3b (opsonin) and C3a (anaphylatoxin). The activation pathways converge on a common terminal pathway with proteolytic activation of C5, which results in the formation of C5a (anaphylatoxin) and C5b. The latter forms the nucleus of the C5b-9 complex, which either remains in its soluble form (sC5b-9) or assembles in the membrane as the membrane attack complex (MAC). Complement activation is regulated by numerous inhibitors and at various levels of the cascade. Ab, antibody; Ag, antigen; CR1, complement receptor 1; C1INH, C1 inhibitor; C4BP, C4b-binding protein; DAF, complement-decay accelerating factor; MASP1, mannan-binding lectin serine protease 1; MBL, mannose-binding lectin; MCP membrane cofactor protein. Reproduced from reference (10) with permission from Springer Nature Limited.



1.3.1 Adaptive immune system cell types

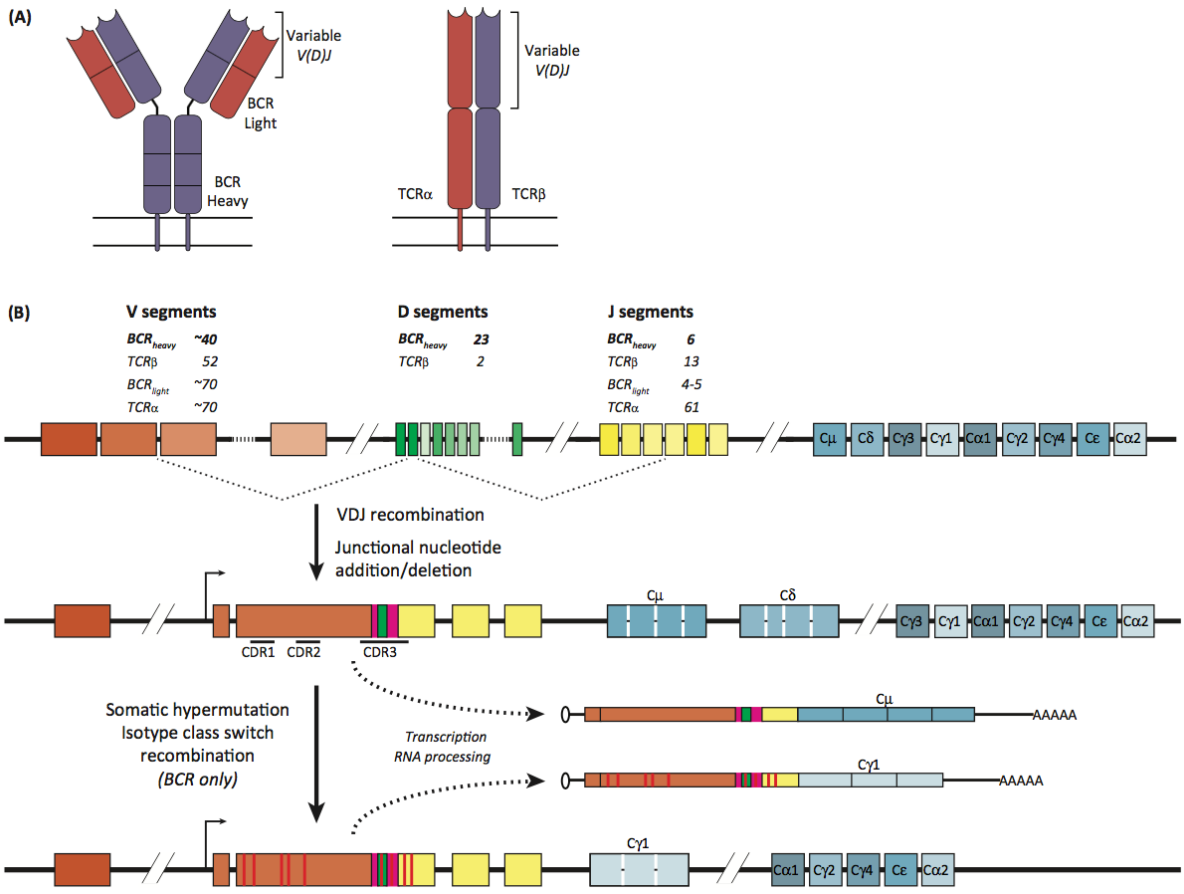
The main adaptive immune system cell types are T and B lymphocytes, which originate from haematopoietic precursors in the bone marrow (BM). Early T lineage progenitors migrate to the thymus during their development, whereas B cell progenitors remain in the BM. Lymphocyte activation and differentiation into mature effector cells occur at peripheral sites such as lymph nodes, spleen and mucosal- or gut-associated lymphoid tissues (MALT/GALT). The sites of lymphocyte development (bone marrow, thymus) are termed primary lymphoid organs and sites of activation are termed secondary lymphoid organs. The majority of lymphocytes are found in lymphoid organs, but migrate between them through blood and lymph (3, 7).

1.3.2 Adaptive immune system pathogen recognition mechanisms

As described earlier, the key feature of adaptive immune pathogen sensing mechanisms is the presence of a wide variety of highly specific antigen receptors, generated during lymphocyte development through random rearrangement of a small number of genes (V, D and J segments, see Figure 1-2), coupled with somatic hypermutation (3). Lymphocytes with "self-reactive" receptors are deleted from the repertoire during development. Both B cell receptors (BCRs) and T cell receptors (TCRs) are expressed on the lymphocyte surface and each lymphocyte has multiple copies of the same receptor, rendering them mono-specific (3). After appropriate stimulation with cognate Ag, lymphocytes undergo rapid clonal proliferation and differentiation into effector cells (detailed below). Over time, the repertoire of BCRs and TCRs therefore undergoes clonal selection, with expansion of populations recognising previously encountered antigens (1).

Figure 1-2 Diversification of lymphocyte receptor repertoire

A – BCRs and TCRs have a similar structure. Each contains two distinct subunit chains (BCR: light and heavy chains); TCR (α and β chains). The variable regions of each chain (encoded by V, D, and J gene segments) form the antigen binding surface. **B** – diversification of antigen receptors is primarily established through rearrangement of V, D and J gene segments during lymphocyte development. A schematic of the BCR heavy chain is shown, but TCR mechanisms are similar, with exception of somatic hypermutation and class-switch recombination, which are mechanisms of BCR-specific secondary diversification. BCR: B cell receptor; TCR: T cell receptor; V, D and J: Variable, Diversity and Joining gene segments. Reproduced from (11) with permission from Elsevier.



1.3.2.1 The B cell receptor

The BCR is a surface immunoglobulin or antibody – a Y shaped, flexible molecule made up of 2 heavy and 2 light chains (see Figure 1-2 A). The type of heavy chain defines antibody class and determines function (summarised Table 1-4). Both types of chains have constant and variable regions, the latter of which is the antigen binding site (3), recognising the 3-dimensional conformation of antigenic epitopes (7).

Table 1-4 Summary of antibody class and function

Ig: immunoglobulin. Adapted from reference (3) under the Creative Commons Licence.

Immunoglobulin class	Function
IgG (subclasses: IgG1-4)	Major form of circulating antibody Secreted during secondary response
IgA (subclasses: IgA1,2)	Major form of antibody in mucosal immunity
IgM	Secreted early during primary response
IgE	Triggers immediate allergic reactions
IgD	Exact function unclear

1.3.2.2 The T cell receptor

The TCR consists of 2 chains (most commonly α and β, see Figure 1-2 A) and is associated with a membrane protein complex that propagates downstream signalling after TCR ligation, collectively known as cluster of differentiation (CD) 3 (12), which is frequently used as a lineage marker for T cells in phenotyping studies.

1.3.2.3 TCRs only recognise antigen presented with major histocompatibility molecules

In contrast to the BCR, the TCR recognises linear antigenic peptides and only in combination with a major histocompatibility (MHC) molecule. As such, for TCR ligation to occur, the antigen first needs to be broken down by specialised antigen-processing cells (APCs) present at the site of injury/microbial invasion. Dendritic cells (DCs) are specialist APCs that fulfil this role (see Figure 1-3). Tissue-resident DCs actively internalise microbial products in their environment, presenting antigenic peptides on their cell surface in combination with either class I or class II MHC molecules. In the context of infection/inflammation, after "antigen capture" DCs migrate to secondary lymphoid organs where they interact with T lymphocytes (3).

1.3.2.4 Costimulatory signals are required for T cell activation

In contrast to pathogen sensing mechanisms in the innate immune system, T cells require additional information about the antigen they recognise before activation and differentiation occurs (1), as if to confirm that the antigen is truly associated with a pathological process (summarised in Figure 1-4). This is delivered through a number of co-stimulatory signals, in particular the ligation of T cell CD28 with APC-derived CD80 or CD86. Other signals include interaction of T cell CD27, OX-40 and CD30 with their respective APC-derived ligands CD70, OX-40L and CD30L. The expression of these ligands is only induced on DCs after pathogen encounter, hence giving a distinct "action is needed" signal to cognate T cells (12). In the absence of co-stimulation, TCR ligation renders T lymphocytes anergic and unable to respond to subsequent stimuli. T cells also

express a number of inhibitory receptors that enable fine-tuning of the T cell response e.g. cytotoxic T lymphocyte antigen (CTLA)-4, which competitively inhibits CD80/86 binding to CD28 and also directly inhibits signalling downstream of the TCR (12).

Costimulatory signals and the production of various soluble mediators after pathogen encounter that shape the differentiation of $CD4^+$ T cells (described later) are ways that the innate immune system exerts control over the type of adaptive immune effector response that is induced (1, 6).

Figure 1-3 Antigen presentation to T cells by dendritic cells.

Antigens presented by dendritic cells in combination with MHC Class I molecules activate cognate CD8+ T cells – this can occur either through direct presentation of intracellular antigen by DCs or through cross-priming, which allows presentation of exogenous antigens to CD8+ T cells. Activation of cognate CD4+ T cells occurs after presentation of antigen combined with MHC Class II molecules. CD: cluster of differentiation; MHC: major histocompatibility complex. Reproduced from reference (13) with permission from Springer Nature Limited.

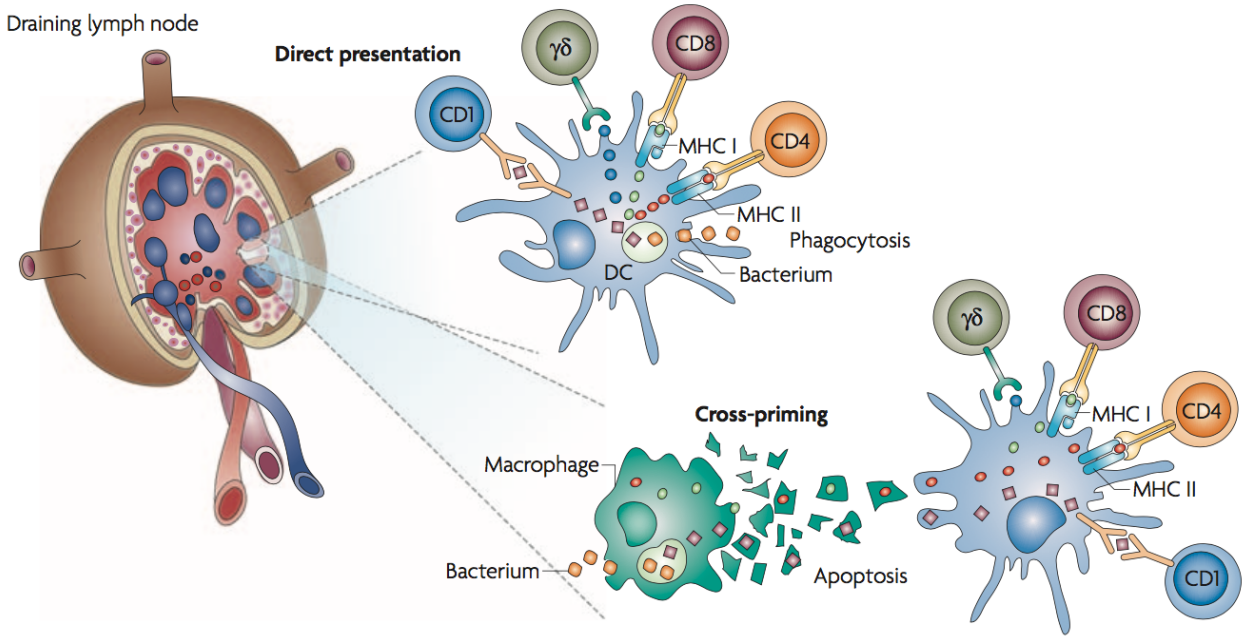
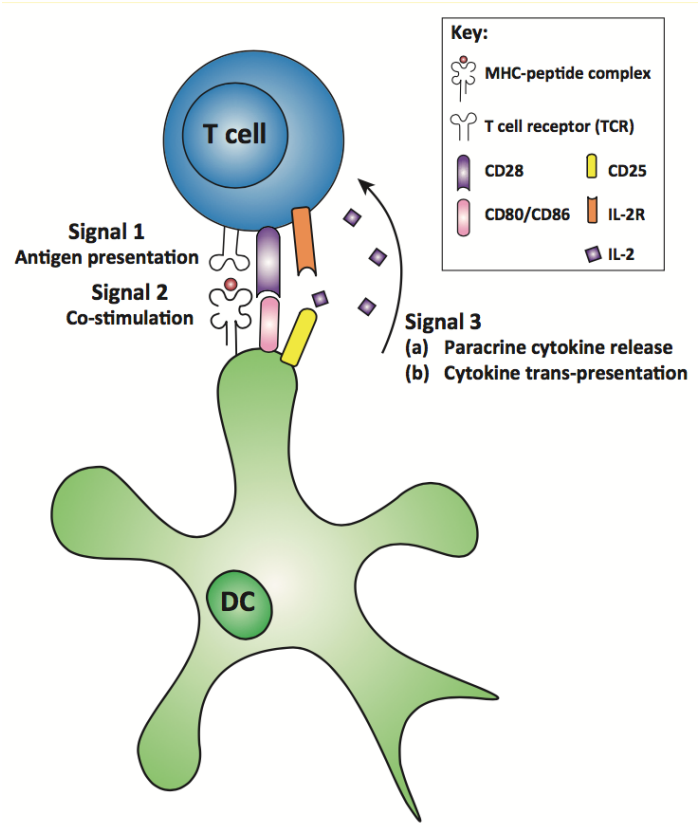


Figure 1-4 Signals leading to induction of T cell activation and differentiation.

Recognition of antigen presented by APCs by naïve T cells represents the first signal, which is followed by ligation of costimulatory molecules e.g. CD28 by APC-derived CD80/86 (signal 2), necessary for full T cell activation. Finally, cytokines released by immune cells involved in the pathogen response enhance cellular expansion and induce differentiation into specific effector phenotypes (signal 3). MHC: major histocompatibility complex; CD: cluster of differentiation; TCR: T cell receptor; IL: interleukin; IL-2R: interleukin 2 receptor; DC: dendritic cell. Reproduced from reference (14) under the terms of the Creative Commons Attribution Licence.



1.3.3 T lymphocyte effector functions

T lymphocyte populations are primarily distinguished by surface expression of CD4 and CD8 molecules. The canonical function of $CD8^+$ T cells is cytotoxicity. Naive $CD8^+$ T cells recognise antigen bound only to class I MHC molecules, which are constitutively

expressed on all nucleated cells. $CD8^+$ T cell effectors are the main players in the adaptive response to viral infections. Activated $CD8^+$ T cells directly induce target cell death by perforin-mediated intracellular delivery of pro-apoptotic proteases (granzymes) and can also inhibit intracellular pathogen replication through the actions of secreted cytokines including Type 1 interferons (IFN) (3).

Activated $CD4^+$ T cells secrete a number of soluble mediators (cytokines) that help to both co-ordinate ("help") and regulate the wider immune response. Naïve $CD4^+$ T cells interact with cognate antigen bound to class II MHC molecules, the expression of which is restricted to immune cells (3, 7, 12).

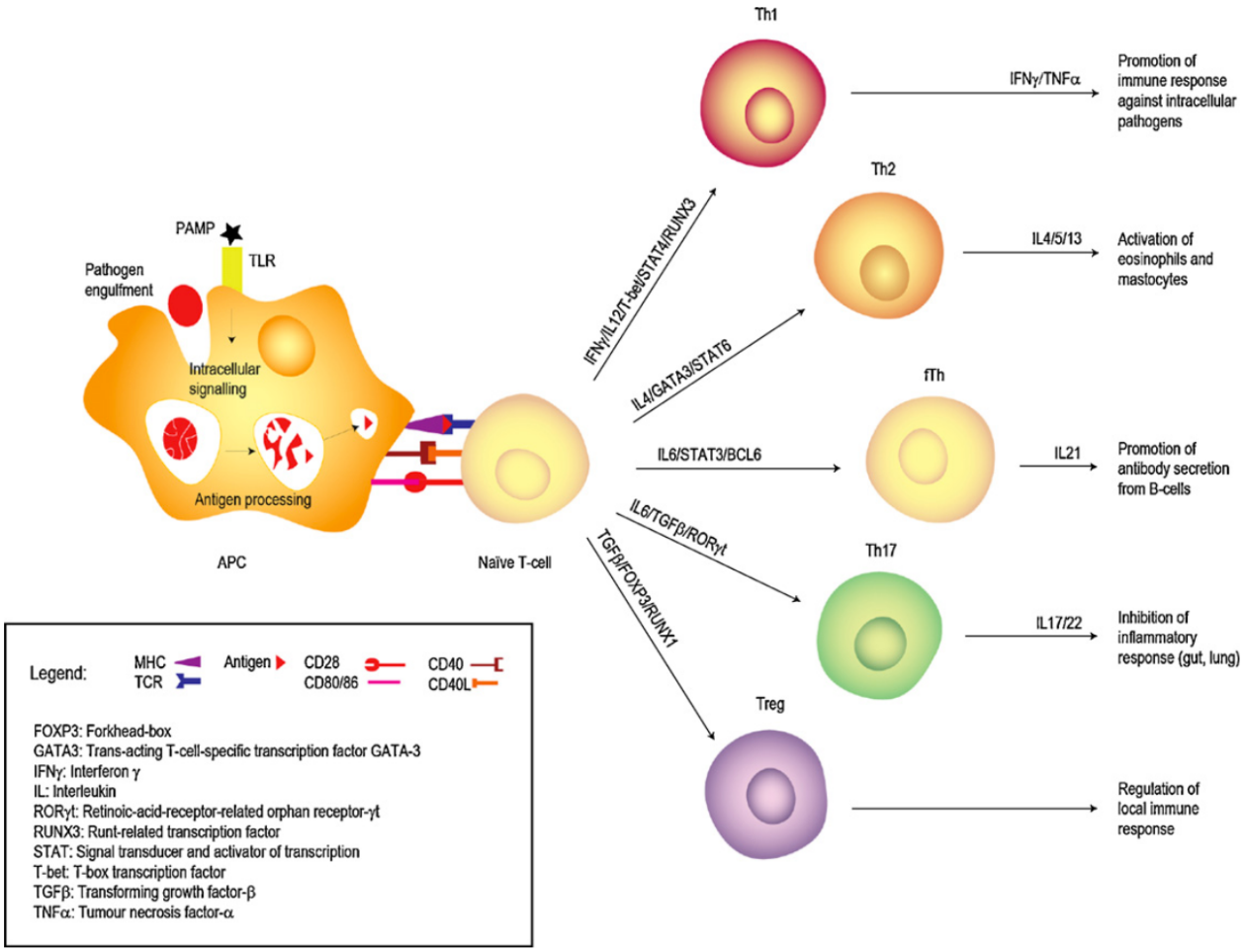
Current understanding of the diversity of the $CD4^+$ T cell effector responses is summarised in Figure 1-5. The type of effector $CD4^+$ T cell phenotype induced depends on the context of antigen encounter and the class of pathogen they recognise. Differentiation of naïve $CD4^+$ T cells into various effector phenotypes is largely directed by cytokines released by innate immune cells (including DCs) during initial pathogen recognition, which control the expression of transcriptional master regulators including T-bet, GATA-3 and RORγT (3).

Individual $CD4^+$ T helper (Th) phenotypes are characterised by a distinct pattern of cytokine secretion. For example, Th1 cells play a key role in defense against intracellular bacteria and protozoa through secretion of IFN-γ and TNF-α. The innate pathogen recognition mechanisms for these microorganisms lead to the production of IL-12 by DCs, which drive differentiation of CD4 T cells into the Th1 phenotype through activation of the transcription factor T-bet (3). It has recently been shown that Th subsets can also be

distinguished by their surface expression of chemokine receptors, including CCR4, CCR6 and CXCR3 (15, 16).

Figure 1-5 CD4+ T cell differentiation into effector phenotypes.

T cell-APC interaction and the different cytokines produced during the immune response to various pathogens stimulate CD4+ T cell differentiation into a number of effector phenotypes. T-helper (Th) 1 cells promote immune responses directed against intracellular pathogens and secrete mainly interferon (IFN) γ and tumour necrosis factor (TNF) α. Th2 cells are involved in helminth defence and secrete interleukins (IL) 4, 5 and 13. Th17 cells secrete IL-17 and IL-22, regulating local immune responses and are involved in autoimmunity. Follicular Th cells (fTh) interact with B cells to stimulate robust antibody production. Regulatory T cells (Treg) limit the extent of immune/inflammatory responses. Reproduced from reference (3) with permission from Elsevier.



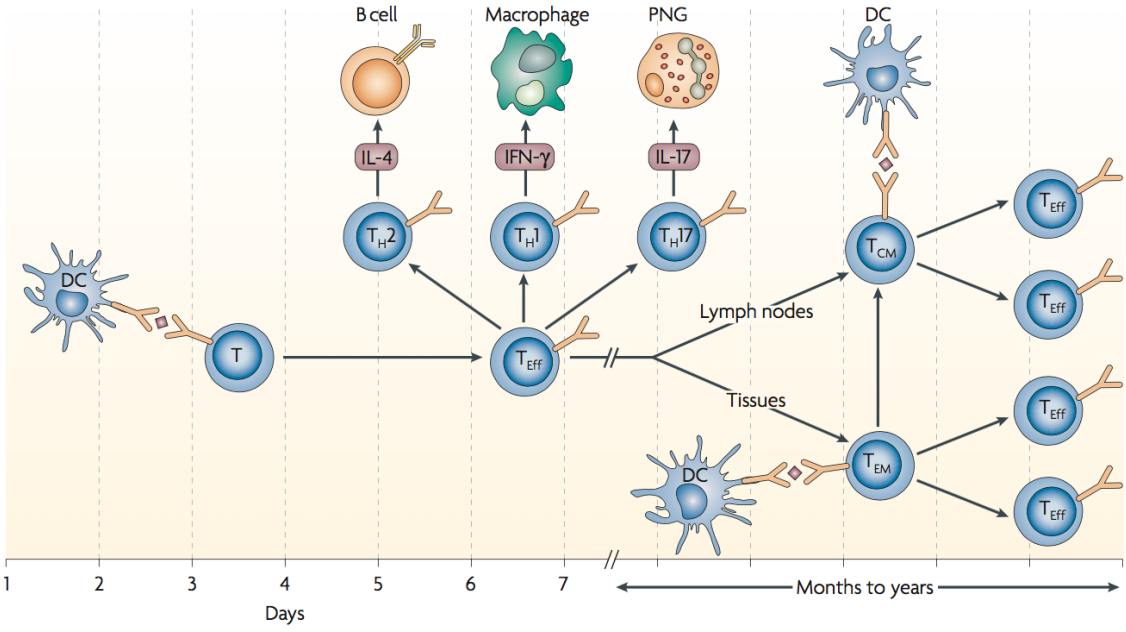
1.3.4 Generation of T cell memory

T cells downregulate their surface expression of CD45RA after encounter with their cognate antigen and this marker is frequently used to distinguish memory from naïve T cells (17). Once T cell activation has occurred, clonally expanded antigen-specific CD4 and CD8 effectors migrate from secondary lymphoid organs to the site of infection, where they exert their functions.

The primary T cell response typically peaks at 7-15 days after antigen exposure (summarised in Figure 1-6), roughly corresponding to the eradication of pathogen by a successful immune response (12). This is followed by a marked contraction of antigen-specific T cells, leaving behind a relatively small pool of long-lived memory T cells. For both $CD4^+$ and $CD8^+$ T cells, memory cells can be divided into 2 main subclasses: central memory (T_{CM}) and effector memory (T_{EM}) (18). T_{CM} cells have an enhanced potential for proliferation upon re-exposure to their cognate antigen and express high levels of the secondary lymphoid organ-homing chemokine receptors CCR7 and CD62L. In contrast, T_{EM} cells have limited proliferative capacity after antigen re-encounter, but can rapidly mount effector functions e.g. granzyme and IFNγ secretion. T_{EM} cells lack the surface expression of CCR7 and CD62L and are, therefore, preferentially trafficked through non-lymphoid tissues to peripheral sites of antigen encounter. A subset of T_{EM} cells re-expressing the marker CD45RA (usually associated with naïve T cells) - T_{EMRA} - are expanded with chronic viral infections e.g. cytomegalovirus (detailed later in this chapter). Initially thought to represent terminally differentiated, non-functional "exhausted" T cells, T_{EMRA} exhibit potent cytotoxic effector activity (19).

Figure 1-6 Generation of T cell memory.

Effector T cells (T_{Eff}) responding to antigen presented by dendritic cells (DCs) differentiate into various T helper (T_H) subsets, examples of which are shown. Two main T cell memory subsets also develop: central memory (T_{CM}) T cells that home to lymph nodes and effector memory (T_{EM}) cells that migrate through non-lymphoid tissues. Memory T cells replicate and differentiate into T_{EFF} populations upon antigen re-encounter. IL: interleukin; IFN: interferon; PNG: polymorphonuclear granulocyte. Reproduced from reference (13) with permission from Springer Nature Limited.



1.3.5 Regulatory T cells

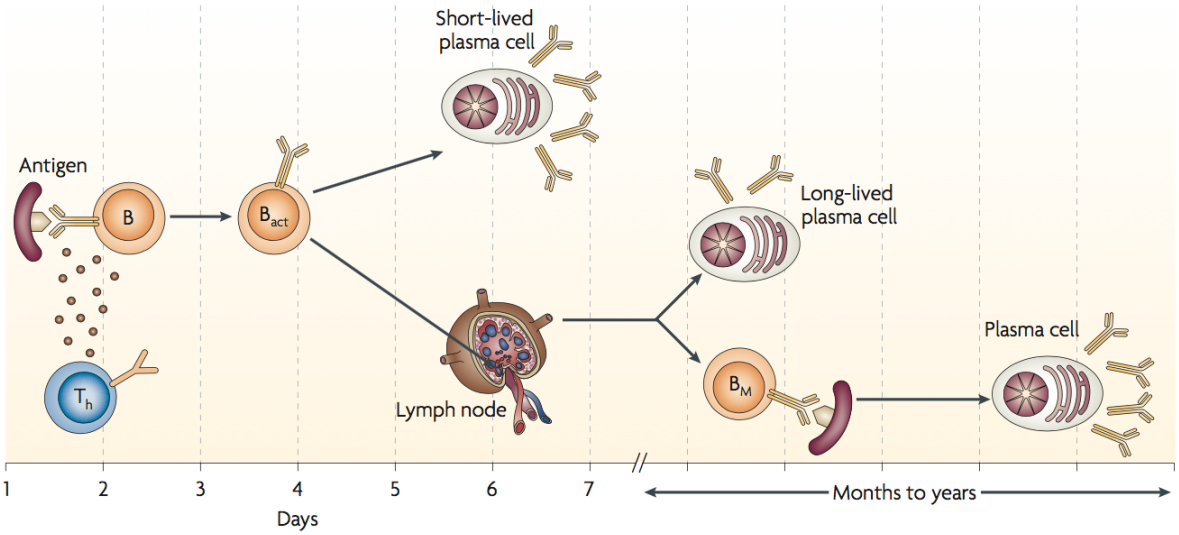
Regulatory T cells (T_{regs} – $CD25^+FoxP3^+$ CD4 T cells) play an important role in immune tolerance by limiting the extent of the immune response and associated tissue damage through inhibition of the activity of APCs and effector lymphocytes (particularly Th cells) (3, 20).

1.3.6 B lymphocyte effector mechanisms

The product of B lymphocytes – antibody - can neutralise antigen by preventing its binding to specific cellular targets and is able to recruit additional effector mechanisms for pathogen clearance through opsonisation of microbes and activation of complement (3). After activation through the BCR, naïve B cells differentiate into both antibody secreting cells (ASCs), termed plasma cells, and high-affinity memory B cells (summarised in Figure 1-7).

Figure 1-7 B cell differentiation and generation of memory.

B cells activated by cognate antigen (B_{act}) and helped by T cells (T_h) differentiate into short-lived plasma cells, which produce the first wave of antibody (Ab) targeting pathogens. Germinal centre reactions involving B_{act} cells in secondary lymphoid organs (e.g. lymph nodes) generate long-lived plasma cells (which continuously secrete Ab) and high-affinity memory B cells (B_M) that efficiently differentiate into high-affinity antibody secreting plasma cells upon antigen re-encounter. Reproduced from reference (13) with permission from Springer Nature Limited.



1.3.7 Antigen-naïve B cell subsets

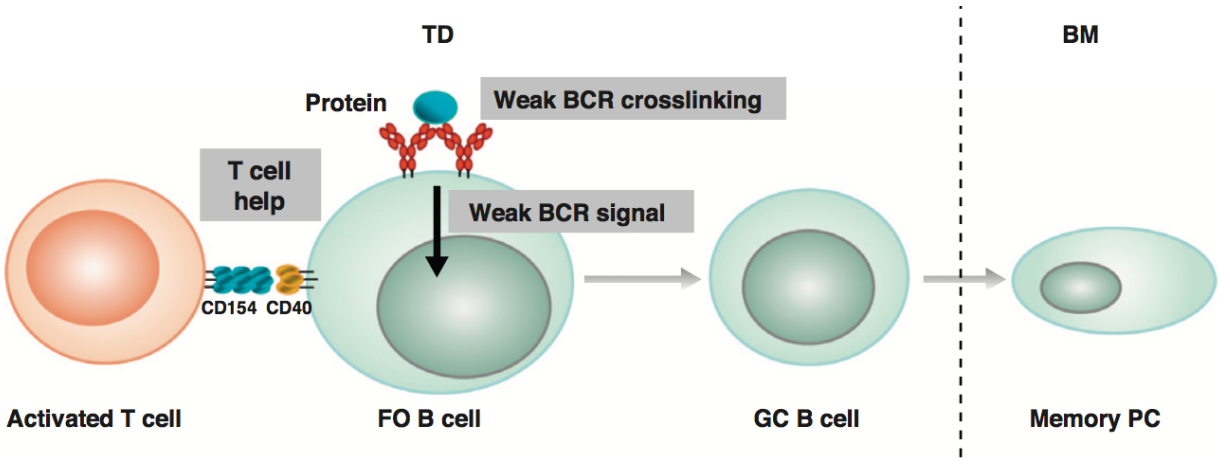
Several naïve B cell subsets exist: follicular B cells (present in secondary lymphoid tissues), marginal zone (MZ) B cells (localised to splenic MZ) and B1 B cells, found in serous cavities including peritoneum and pleura (21, 22). Naïve B cells initially express immunoglobulins of the IgM and IgD isotypes, but are able to switch to secretion of IgG, IgA and IgE after activation.

1.3.8 T-dependent B cell activation and the germinal centre response

High affinity, class switched antibody is produced through activation of follicular B cells and their interaction with helper T cells in specialised cellular structures in secondary lymphoid organs, termed germinal centres (GCs) (21) – summarised in Figure 1-8. This process is dependent on the APC function of B cells: naive B cells that encounter their cognate antigen internalise and process it, and then present antigenic peptide fragments in conjunction with class II MHC to $CD4^+$ T cells. In GCs, activated B cells undergo somatic hypermutation of their BCR genes, immunoglobulin class switch recombination (CSR or isotype switching), affinity maturation and clonal expansion (6, 23). CSR largely occurs in response to 2 signals: binding of follicular T helper cell (Tfh) CD40L to B cell-derived CD40 and secretion of distinct sets of cytokines (e.g. from Th1 and Th2 cells), which help to direct isotype and subclass selection (3, 6). Antigens eliciting such a response are termed T-dependent (TD) antigens.

Figure 1-8 T-dependent (TD) B cell activation.

Follicular (FO) B cells are activated by relatively weak BCR crosslinking and costimulatory signals provided by cognate CD4+ T cells. These B cells first differentiate into germinal centre (GC) B cells and undergo class switch recombination and clonal selection, followed by generation of memory plasma cells (PC) or memory B cells. BCR: B cell receptor; CD: cluster of differentiation; BM: bone marrow. Reproduced from reference (21) with permission from Elsevier.



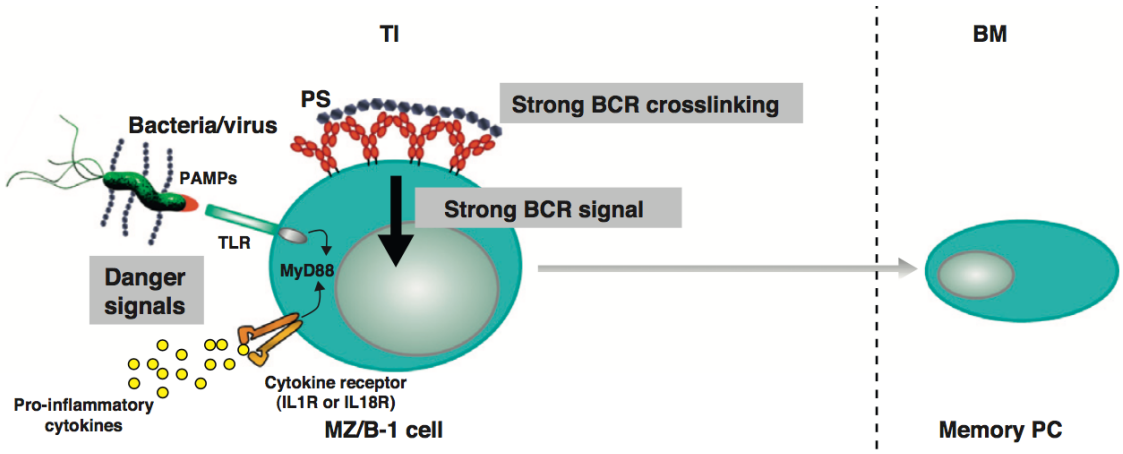
1.3.9 T-independent B cell activation and the extra-follicular response

Antibody production and generation of Ag-specific B cell memory can also occur in the absence of T cell help (see Figure 1-9). Such "T-independent" (TI) antigens can activate B cells either by concomitant ligation of TLRs and the BCR (e.g. lipopolysaccharide (LPS), flagellin – described as TI-1 antigens) or through extensive BCR crosslinking by a highly repetitive arrangement of antigenic epitopes, as is seen with bacterial polysaccharides (PS, described as TI-2 antigens) (1, 21). MZ and B1 B cells play a key role in responses to TI antigens (21). This extra-follicular B cell response results in the transient generation of low affinity antibody, most commonly of the IgM isotype, although class switch can also

occur as a result of canonical & non-canonical NF-κB signalling-induced activation-induced cytidine deaminase (AID) expression that follows TLR ligation (6).

Figure 1-9 T-independent (TI) B cell activation

Marginal zone (MZ) or B-1 B cells are stimulated by strong crosslinking of the BCR (B cell receptor) by highly repetitive TI antigens e.g. polysaccharides (PS), together with accessory danger signals delivered via TLR (Toll-like receptor) stimulation by PAMPs (pathogen associated molecular patterns) or pro-inflammatory cytokines. Memory plasma cells are generated as a result of this extrafollicular response and home to bone marrow (BM) niches. Reproduced from reference (21) with permission from Elsevier.



1.3.10 Generation of B cell memory

ASCs "fight from a distance" - most plasma cells migrate to bone marrow (mediated by upregulation of the chemokine receptor CXCR4) to reside in uniquely supportive niches, where they produce large amounts of antibody that is carried in the bloodstream and by other bodily fluids to the site of infection (3, 23). The lifespan of plasma cells varies.

Short-lived (days) plasma cells are responsible for the acute phase of antibody secretion, whereas long-lived (years) plasma cells maintain circulating levels of antibody after pathogen clearance. Although both TI and TD antigens give rise to both short and long-lived plasma cells, the effector quality of the latter is far superior from TD mechanisms than from TI (23).

Memory B cells are largely generated from GC reactions and rapidly differentiate into high-affinity ASCs following repeat exposure to their cognate antigen, thus achieving faster pathogen clearance than the primary response. Different subtypes of memory B cells are identified by their different Ig isotypes (e.g. IgM and IgG or class-switched memory B cells) and levels of expression of costimulatory molecules (24). Memory B cells can differentiate from responses to TI antigen, but appear to lack the canonical functions of memory - that is, enhanced sensitivity to Ag re-stimulation and lifespan compared to unstimulated cells (21). Importantly, reduced TI memory B cell responses to re-challenge with their cognate TI antigen has previously been reported (21, 25). This may be a regulatory response to antigens that biodegrade slowly e.g. polysaccharides (21), but the full biological significance of this remains unclear.

1.3.11 Regulatory B cells

A regulatory subset of B cells (B_{regs}) plays an integral role in maintaining immune homeostasis, through secretion of the cytokines IL-10, TGF-β and IL-35. B_{regs} direct T cell differentiation towards a T_{reg} phenotype, inhibit the differentiation of Th1 and Th17 phenotypes and induce anergy in $CD8^+$ effectors, thus limiting tissue damage due to pathogen-induced inflammation (26).

1.4 Vaccination

The principle of vaccination is to utilise the ability of the adaptive immune system to generate long-lived antigen-specific memory (with enhanced responses upon antigen re-exposure) by using an agent that closely mimics natural infection with the pathogen of interest, but does not cause disease. The ideal vaccine closely mimics natural innate & adaptive immune activation to induce long-lasting humoral and cellular memory, without eliciting toxicity e.g. inflammation to a degree that would be damaging to the host.

1.4.1 Types of vaccines currently used in humans

Several broad groups of vaccines exist. Live attenuated vaccines (LAVs, consisting of weakened versions of pathogens e.g. smallpox, varicella, measles) generally elicit the most robust memory responses (even after a single vaccination) as they are the closest mimic to natural infection (27).

"Non-live" vaccines use inactivated pathogens, antigenic protein subunits (e.g. HBV), inactivated toxins (toxoids e.g. DT/TT) or bacterial polysaccharides e.g. the 23-valent pneumococcal polysaccharide vaccine (PPV23) (27). Glycoconjugate vaccines, where bacterial polysaccharides (TI antigens that, alone, elicit weak memory responses) are covalently bound to a protein that engages with and stimulates T cell responses, thus enhancing the quality of the polysaccharide response, are an important tool used in eliciting immune responses to TI antigens in the immature immune systems of infants and young children (28).

1.4.2 Vaccine adjuvants

As “non-live” vaccines generally elicit weaker immune responses compared to LAVs, they typically contain other substances that enhance the quality and diversity of the elicited immune response – adjuvants. The 3 common licensed adjuvants are summarised in Table 1-5. Adjuvant effects are largely elicited through activation of components of the innate immune system e.g. by enhancing pathogen recognition and presentation, stimulation of cytokine and chemokine secretion with resultant recruitment of immune cells to the site of vaccine delivery, together with inflammasome activation (29). In addition, adjuvants can mimic the different immune effector polarisation signals required for effective clearance of different types of pathogens (e.g. virus, bacteria).

1.4.3 Assessing vaccine response

Responses to vaccination are generally evaluated by the magnitude of the elicited humoral adaptive response (30, 31). Although vaccine induction of neutralising antigen-specific antibody is thought to be important in preventing disease (particularly for viruses), the amount of antibody required for this protection remains unclear for many pathogens. More recently, the polyfunctionality of antigen-specific T cells elicited by vaccination has been proposed as a key cellular read-out for the quality of vaccine response, particularly to viral antigens e.g. influenza (32). This is supported by the observation that live attenuated influenza vaccines can elicit far superior protection against infection, but with a lower magnitude of antibody response to their inactivated vaccine counterparts (33).

Table 1-5 Summary of common vaccine adjuvants licensed for use in humans.

DT: diphtheria toxoid; TT: tetanus toxoid; Hib: *Haemophilus influenzae b*; Th: T helper; NLRP3: nucleotide-binding domain leucine-rich repeat family, pyrin domain containing 3; TLR: Toll-like receptor; DC: dendritic cell; APC: antigen-presenting cell. Adapted from reference (29) under the Creative Commons Licence.

Adjuvant	Mechanism of Action	Effect on immune response	Licensed vaccines
Alum	Induction of local cytokine and chemokine secretion Immune cell recruitment (monocytes, macrophages, eosinophils) Enhanced antigen presentation NLRP3 inflammasome activation **Independent of TLR signalling**	Enhanced antibody production Polarisation towards Th2 effector responses	Many vaccines including DT/TT, Hib, Hepatitis B (Engerix B)
MF59	Induction of local cytokine and chemokine secretion Immune cell recruitment (neutrophils, monocytes, macrophages) Enhanced antigen uptake by APCs Enhanced trafficking of antigen-loaded neutrophils and monocytes in draining lymph nodes **Independent of TLR signalling**	Balanced Th1 & Th2 responses	Influenza vaccine (Fluad), H1N1 pandemic vaccines
AS04	Induction of local cytokine and chemokine secretion Immune cell recruitment (DCs, monocytes) Enhanced trafficking of antigen-loaded DCs and monocytes in draining lymph nodes **TLR4 signalling to activate APCs**	Enhanced antibody production Polarisation towards Th1 effector responses	Human papilloma virus (Cervarix™)

1.5 Common respiratory pathogens in humans

In the next section of this Chapter, I will briefly describe two common respiratory pathogens for which vaccines are commonly used in adults to reduce morbidity and mortality: influenza virus and *Streptococcus pneumoniae*.

1.6 Influenza virus

Influenza viruses are negative strand RNA viruses of the Orthomyxoviridae family and the 3 main types known to cause human disease are summarised in Table 1-6 (34). Influenza A viruses are, to date, the only types to have caused pandemics (34), and, together with B viruses, are responsible for the majority of clinically significant influenza disease in humans (35).

1.6.1 Epidemiology of influenza virus infections

In climates with seasonal variation (away from the equator), influenza virus activity is increased during the colder months e.g. typically from December to March in the northern hemisphere (34). This variation has coined the term “seasonal influenza”. Although clinically significant influenza pandemics occur outside this variation, their description is outside the scope of this Chapter. Seasonal influenza represents an important global health problem. Influenza-related mortality is estimated at 4.0 to 8.8 per 100 000 individuals per year, which equates to 290 000 – 645 000 seasonal influenza-associated respiratory deaths worldwide (36).

Table 1-6 Summary of influenza virus types known to cause human disease.

HEF: haemagglutinin-esterase-fusion protein. Table compiled from references (34, 35) under the Creative Commons Licence.

Influenza type	Subtypes	Main reservoir	At risk groups
A	Classification based on structure of surface haemagglutinin (H1-18) and neuraminidase (N1-11) antigens Only 2 subtypes currently circulating: H1N1 and H3N2. Nomenclature also includes natural host species, geographical origin, year of isolation and strain number	Acquatic birds, pigs, horses, seals.	All ages; severe disease in immunocompromised individuals
B	Classification based on haemagglutinin glycoprotein structure	Humans	Children
C	Only one glycoprotein (HEF)	Humans	All ages, but generally causes mild illness

1.6.2 Clinical features of influenza infection

Influenza virus generally causes an acute respiratory disease and

Figure 1-10 summarises the main clinical symptoms and complications of influenza virus infection. Virus transmission is typically through respiratory droplets and direct contact with infected (virus-shedding) individuals, with the incubation period lasting between 1 and 4 days. Complications are more common in young children, healthy adults over 65 years of age and immunocompromised adults e.g. with chronic diseases including chronic respiratory & kidney disease (discussed later).

Figure 1-10 Summary of clinical symptoms, signs and complications of influenza virus infection.

Symptoms and signs denoted by blue symbols, complications denoted by orange symbols.

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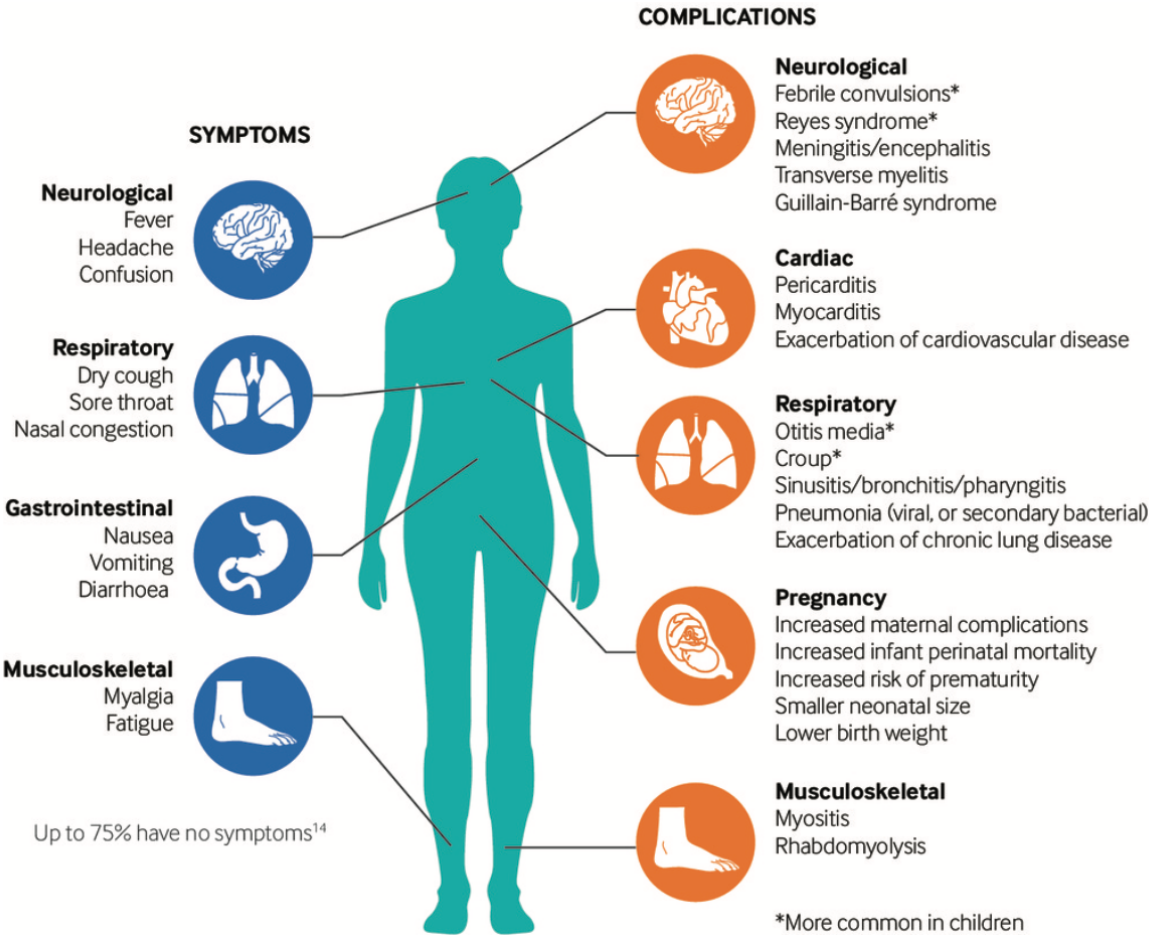
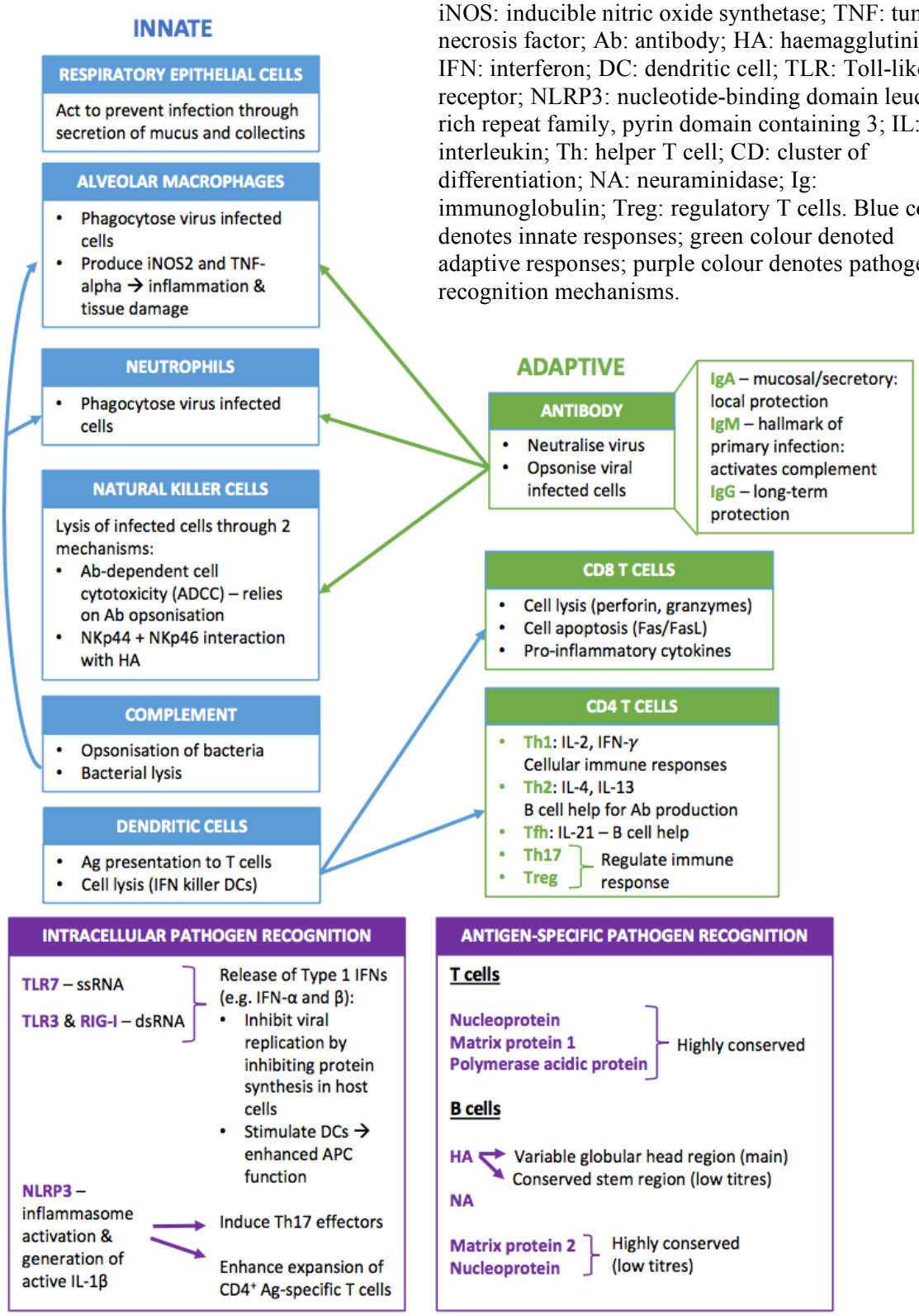


1.6.3 Immune responses to influenza infection

Figure 1-11 summarises the immune response to natural influenza infection

Figure 1-11 Summary of immune responses to natural influenza virus infection.

Compiled from (37).



iNOS: inducible nitric oxide synthetase; TNF: tumour necrosis factor; Ab: antibody; HA: haemagglutinin; IFN: interferon; DC: dendritic cell; TLR: Toll-like receptor; NLRP3: nucleotide-binding domain leucine-rich repeat family, pyrin domain containing 3; IL: interleukin; Th: helper T cell; CD: cluster of differentiation; NA: neuraminidase; Ig: immunoglobulin; Treg: regulatory T cells. Blue colour denotes innate responses; green colour denoted adaptive responses; purple colour denotes pathogen recognition mechanisms.

The influenza virus attaches to epithelial cells of the upper respiratory tract through haemagglutinin (HA) binding to sialic-acid residues on host membrane glycoproteins, which facilitates subsequent viral entry. Influenza neuraminidase (NA) is essential for intracellular viral propagation and release (34).

1.6.4 Vaccination strategies against influenza

Vaccination represents the most cost-effective way to prevent influenza disease and its associated complications. Current seasonal influenza vaccines are usually trivalent (derived from 2 influenza A strains and 1 B strain) and contain either live attenuated viruses or inactivated influenza antigens (summarised in Table 1-7).

Table 1-7 Summary of common types of influenza vaccines.

Ig: immunoglobulin; HA: haemagglutinin; NA: neuraminidase. Compiled from reference (38).

Vaccine type	Antigenic components	Route of administration	Patient group	Immune responses
Live attenuated influenza vaccine (LAIV)	Live attenuated influenza virus (trivalent) – usually propagated in eggs.	Intranasal (mimics natural infection route)	Children and adults (ages 2-49 years)	Robust mucosal IgA response Cell-mediated response
Trivalent inactivated influenza vaccine (TIV)	Virus initially propagated in eggs. Various compositions and structural organisation: • Whole inactivated virus • Virosomes (reconstituted viral envelopes consisting of HA, NA and viral phospholipids) • Split antigen (virus particles disrupted by diethyl ether/detergent; viral structural organisation and ssDNA lost) • Subunit antigen (HA and NA proteins only)	Intramuscular	All ages	Neutralising anti-HA and anti-NA antibodies Cell-mediated response less well characterised

Although different methods of virus inactivation or TIV Ag composition may theoretically impact individual vaccine immunogenicity, this has not been borne out in meta-analyses (39, 40). Current vaccination strategies generally induce antibodies against HA and NA, the genes of which exhibit marked antigenic shift and drift. As such, neutralising antibodies generally lack cross-reactivity against non-matching influenza strains (34). Seasonal adjustments to vaccine strain composition are made in order to cope with this problem, based on WHO surveillance of prevalent influenza viruses (41). Table 1-8 summarises the current recommendations for seasonal influenza vaccination in adults.

Table 1-8 Current UK recommendations for influenza vaccination in adults.

COPD: chronic obstructive pulmonary disease; HTN: hypertension; TIA: transient ischaemic attack; BMI: body mass index. Compiled from reference (42).

Category	Examples
All adults aged 65 years or older	
Adults <65 years in clinical risk groups (below)	
Chronic respiratory disease	Asthma requiring use of inhaled or systemic steroids COPD
Chronic heart disease	Congenital heart disease, HTN with cardiac complications, chronic heart failure, ischaemic heart disease requiring treatment or specialist follow-up
Chronic kidney disease	Stages G3-5, nephrotic syndrome, kidney transplantation
Chronic liver disease	Cirrhosis, chronic hepatitis
Chronic neurological disease	Stroke/TIA; clinically vulnerable individuals with cerebral palsy, learning disabilities, multiple sclerosis.
Diabetes Mellitus	All types
Immunosuppression	Chemotherapy recipients, bone marrow transplant, HIV infection at all stages.
Asplenia/splenic dysfunction	Sickle cell disease
Pregnant women	Any stage of pregnancy
Morbid obesity	BMI ≥ 40kg/m^2
Health & social care workers in direct contact with patients/clients	Hospital or long-stay care facility staff, main carers of an elderly person whose welfare may be at risk if carer falls ill, individuals in receipt of carer's allowance.

Recent Cochrane reviews concluded that seasonal inactivated influenza vaccination likely reduces the number of healthy and older (>65) adults that experience influenza infection and influenza-like illness, compared to unvaccinated individuals (43). However, the evidence of benefit in older adults, in particular with regards to influenza complications is limited.

1.7 Streptococcus pneumoniae

Streptococcus pneumoniae (pneumococcus, Pn) is a facultatively anaerobic encapsulated Gram-positive bacterium that frequently colonises the mucosal surfaces of the upper respiratory tract in humans (44). This extracellular pathogen is a highly adapted commensal with nasopharyngeal carriage rates of approximately 10% in adults, but as high as 65% in children (45).

1.7.1 Clinical features of pneumococcal disease

Pneumococci can cause varying degrees of local and systemic infection in its obligate human host through local spread (e.g. otitis media), aspiration (e.g. pneumonia) or bloodstream seeding (e.g. meningitis and/or septicaemia) (45). Indeed, pneumococcus is the most common causative organism of community-acquired pneumonia (CAP) worldwide (accounting for approximately 35% (46)). The term “invasive pneumococcal disease” (IPD) describes the presence of pneumococci in normally sterile sites e.g. blood or cerebrospinal fluid (47). Transmission is generally through respiratory droplet spread during close contact with pneumococcal carriers, but can also occur through fomites, as

pneumococcus can form hardy biofilms on inanimate objects that are resistant to the environment (44).

1.7.2 Epidemiology of pneumococcal disease

The burden of pneumococcal disease is high. Young children (under 5 years old) and older adults (over 65 years) are particularly affected. Estimated annual incidence of CAP in adults is 2-10 per 1000 individuals in western countries, markedly increasing with age (over 50 per 1000 person-years in individuals aged over 85) (47). The incidence of IPD is generally lower (estimates range from 10 to 50 per 100 000 person/years), but the case fatality rate is much higher (up to 30% in IPD versus approximately 10% in individuals hospitalised with CAP), again increasing with older age (47). Importantly, coinfection with pneumococcus is often seen during viral infections e.g. influenza, resulting in increased risk of morbidity (including incidence of IPD) and mortality, together with increased rates of pneumococcal transmission (47).

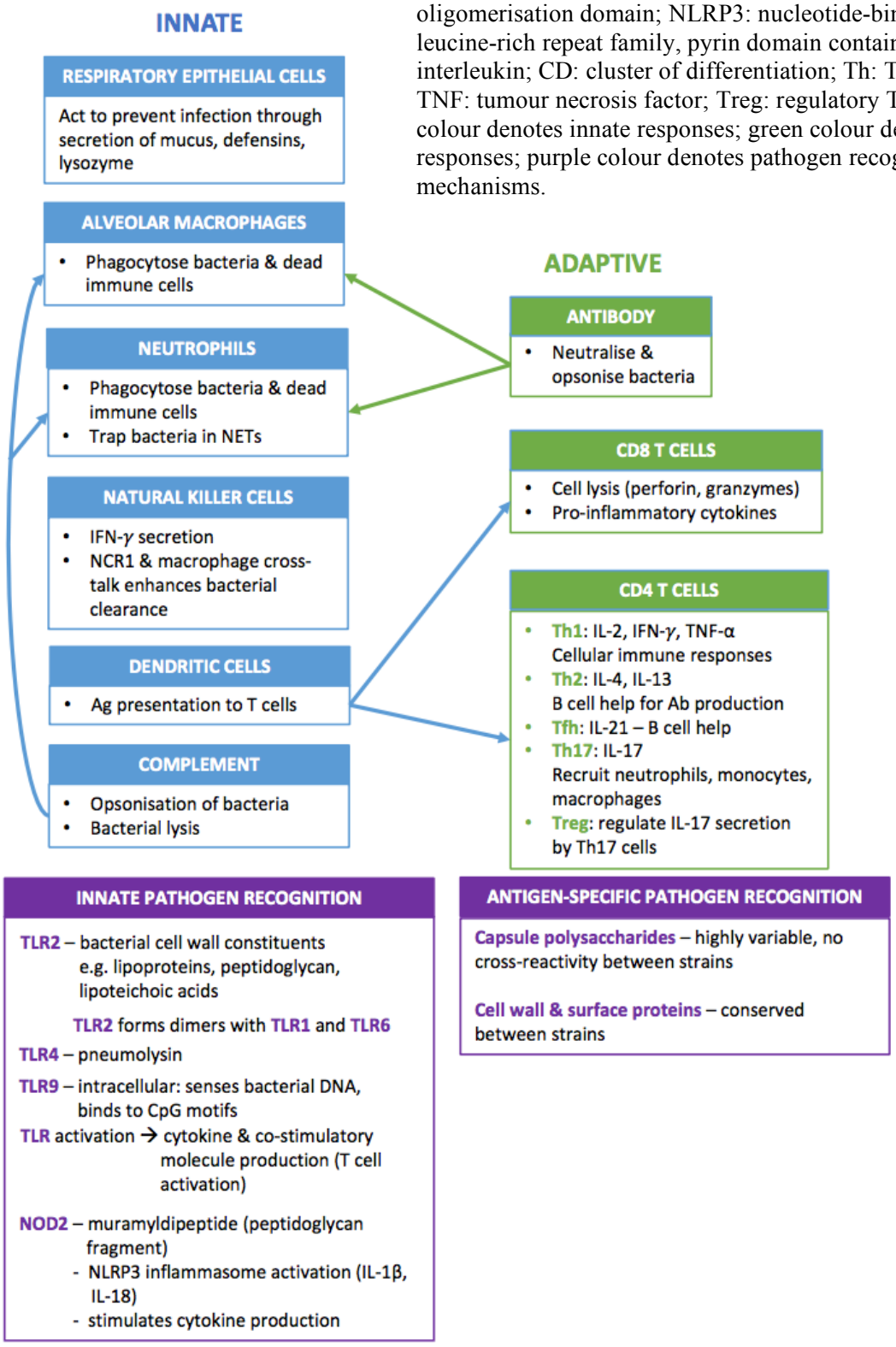
1.7.3 Immune responses to pneumococcal infection

Transformation of pneumococcus from commensal to pathogen mainly depends on the duration of colonisation, which is controlled by 2 main factors: competition with resident flora and host immune response competency (44). Figure 1-12 summarises the host immune response to natural pneumococcal infection.

Figure 1-12 Summary of immune responses to natural infection with *Streptococcus pneumoniae*.

Compiled from references (44, 48, 49).

NET: neutrophil extracellular trap; IFN: interferon; NCR: natural killer cell activating receptor; TLR: Toll-like receptor; DNA: deoxyribonucleic acid; NOD: nucleotide-binding and oligomerisation domain; NLRP3: nucleotide-binding domain leucine-rich repeat family, pyrin domain containing 3; IL: interleukin; CD: cluster of differentiation; Th: T helper cells; TNF: tumour necrosis factor; Treg: regulatory T cells. Blue colour denotes innate responses; green colour denoted adaptive responses; purple colour denotes pathogen recognition mechanisms.



1.7.4 Pneumococcal host evasion and virulence factors

Pneumococcus has a number of virulence and host evasion factors that promote invasion of respiratory epithelial cells and subsequent infection (summarised in Table 1-9).

Table 1-9 Summary of pneumococcal virulence factors and effects.

NET: neutrophil extracellular trap; Ig: immunoglobulin; DNA: deoxyribonucleic acid. Compiled from reference (44).

Virulence factor	Effect/mechanism
Polysaccharide capsule	• Promotes adherence to host epithelial cells • Inhibits pathogen recognition, opsonisation, phagocytosis and NETs entrapment • Evades entrapment by mucus through electrostatic repulsion
Cell wall components e.g. peptidoglycan, lipoteichoic acids, wall teichoic acids.	• Resistance to action of lysozyme • Act as anchors for choline-binding proteins • Induce inflammatory response
Pneumolysin	• Pore-forming toxin causes host cell lysis • Promotes biofilm formation • Inhibits phagocytosis • Induces inflammatory response • Enhances host-to-host transmission
Autolysin	• Promotes nasopharyngeal colonisation due to release of toxins e.g. pneumolysin during cell wall degradation (bacterial autolysis).
Pneumococcal surface proteins e.g. choline-binding proteins, lipoproteins, non-classical proteins	• Promote adherence to host epithelial cells • Inhibit complement binding/activation • Promote resistance to oxidative stress • Induce inflammatory response
Pili	• Promote adherence to host epithelial cells • Inhibit phagocytosis
IgA1 protease	• Breaks down human IgA1 antibody • Inhibits mucosal antibody-mediated defense
Hydrogen peroxide	• Damage to host DNA • Bacteriostatic effect on competition commensals
Pathogenicity islands	• Parts of pathogenic bacterial genome coding for iron-uptake systems & proteins involved in adherence to host cells
Biofilms	• Aggregates of bacteria surrounded by extracellular polysaccharide matrix • Promote bacterial survival e.g. through enhanced gene transfer rates (e.g. to promote antibiotic resistance) and evasion of mucociliary clearance

Of these, the pneumococcal polysaccharide capsule is the most important and is responsible for adherence to host cells, evasion of pathogen recognition, opsonisation and phagocytosis (44). The structure of capsule polysaccharides determines the pneumococcal serotype (over 90 are known) and the type of disease caused in the host e.g. serotype 1 is particularly associated with IPD, whereas 6C and 35B are associated with non-invasive disease (50). The ability of the host to produce antibody directed against specific capsular polysaccharides is a key feature of the host immune defence against pneumococcus (51). However, pneumococci can undergo "serotype switching" through mutations in capsule polysaccharide genes in response to selective pressures from antibiotic and vaccine use, further evading the immune response (44).

1.7.5 Vaccination strategies against pneumococcus

A number of vaccines have been developed in order to reduce the burden of pneumococcal disease in both adults and children – the 23-valent pneumococcal polysaccharide vaccine (PPV23) and pneumococcal conjugate vaccines (PCV) against 7, 10 or 13 serotypes. Current recommendations in the UK advocate the use of PCV13 in the primary childhood immunisation schedule and PPV23 in at risk adults (summarised in Table 1-10) (52). Although immune responses to PCV (TD) are superior to PPV23 (TI), the greater variety of serotypes contained in PPV23 may be an advantage in an adult population (with a mature immune system, able to respond to TI antigens) due to serotype replacement. Serotype replacement has been reported since the introduction of widespread childhood PCV vaccination, with the emergence of severe disease caused by non-vaccine strains e.g. paediatric 19A-associated IPD in the post-PCV7 era (53). The use of multivalent

conjugates in children may lead to the emergence of non-vaccine strains in adults through indirect herd immunity effects (51). Therefore, administration of the same vaccines as are used in the paediatric population may not confer extra benefit in adults.

Table 1-10 Current UK recommendations for PPV23 vaccination in adults.

COPD: chronic obstructive pulmonary disease; HTN: hypertension. Table compiled from reference (52).

Category	Examples
All adults aged 65 years or older	
Adults <65 years in clinical risk groups (below)	
Chronic respiratory disease	Severe asthma requiring repeated use of systemic steroids COPD Bronchiectasis
Chronic heart disease	Congenital heart disease, HTN with cardiac complications, chronic heart failure, ischaemic heart disease requiring treatment or specialist follow-up
Chronic kidney disease	Stages G4-5, dialysis, nephrotic syndrome, kidney transplantation
Chronic liver disease	Cirrhosis, chronic hepatitis
Individuals with cochlear implants / cerebrospinal fluid leaks	Vaccination should not delay cochlear implantation; Cerebrospinal fluid (CSF) leaks include those following trauma/major skull surgery and all CSF shunts.
Diabetes Mellitus	Excluding diet-controlled diabetes.
Immunosuppression	Chemotherapy recipients, bone marrow transplant, HIV infection at all stages.
Asplenia/splenic dysfunction	Sickle cell disease
Occupational risk	Frequent exposure to metal fume e.g. welders

PPV23 is also used as a tool to assess humoral immune function in both children and adults. The American Academy of Allergy, Asthma and Immunology (AAAAI) guidelines for the diagnosis and management of primary immunodeficiency advocate the use of

PPV23 in individuals ages 2-65 as a tool in the diagnosis of antibody deficiencies, in particular selective antibody deficiency (SAD) - characterised by recurrent respiratory infections, normal total immunoglobulin and IgG subclass levels, but poor humoral responses to pneumococcal polysaccharides (54).

1.7.5.1 PPV23 hyporesponsiveness

PPV23 may have limited utility due to hyporesponsiveness reported with repeat administration - defined as the inability of an individual to mount an immune response upon re-exposure to antigen of at least the same or greater magnitude as that seen after primary exposure (51). Although this has been reported in various adult cohorts (55-57), it is not a consistent finding (58-60) and the clinical impact of this remains under debate. Interestingly, hyporesponsiveness is also observed after natural exposure to pneumococcus, with reduced secondary responses in children to serotypes previously found colonising the nasopharynx (57).

Several hypotheses have been postulated for the observed hyporesponsiveness to pneumococcal polysaccharides, including depletion of the memory B cell pool generated following primary exposure to polysaccharides (TI antigen) and chronic immune activation due to antigen persistence (25, 61). Further research is ongoing to discover conserved pneumococcal antigens that could both broaden vaccine coverage and enhance host immune responses (51).

1.8 Immune dysfunction with ageing

Chronological ageing is associated with a decline in the effectiveness of immune responses as evidenced by increased susceptibility to infection (62), together with reduced immune responses to vaccines, including influenza (63-65) and pneumococcus (66, 67). Self-regulatory immune functions are also impaired, leading to a greater incidence of malignancy and autoimmunity (68, 69).

1.8.1 Innate immune system alterations with older age

A number of innate immune system alterations have been described with ageing that could explain these observations (reviewed in (70-72)). For example, age-related reduction in mucin secretion and cilia beat frequency may promote dissemination of respiratory pathogens such as influenza and pneumococcus (44). The phagocytic function of macrophages and neutrophils is impaired with older age (73), with neutrophils also exhibiting reduced killing ability and generation of NETs (74). Aged neutrophils also demonstrate impaired chemotactic accuracy, which, together with impaired migration to sites of infection, may result in overproduction of proteases, leading to increased local inflammation and tissue damage (75). These changes may be partly ascribed to defects in pathogen recognition mechanisms e.g. downregulation of TLR1 expression and reduction of TLR4 function seen with age (76, 77). Both NK cells and DCs have also shown to have globally reduced function with ageing (78).

1.8.2 Adaptive immune system alterations with older age

The efficacy of the adaptive immune response also diminishes with increasing chronological age. Ageing is associated with a contraction of naïve T and B lymphocyte pools due to both a reduction in naïve cell generation (e.g. thymic involution) and/or impaired homeostatic maintenance mechanisms, together with differentiation into memory subsets with antigen experience, which results in the relative accumulation of memory lymphocytes with a restricted TCR/BCR repertoire (78). Numerical reduction of naïve lymphocytes theoretically reduces the capacity of the adaptive immune system to respond to novel antigens. Lymphocyte differentiation results in cell populations with limited replicative capacity (in part due to telomere shortening and accumulation of DNA damage (79)) – also described as "senescent" cells. Ageing is also associated with accumulation of highly differentiated lymphocytes that lose effector function – "exhausted" cells. However, it is increasingly recognised that replicative senescence and functional exhaustion do not go hand in hand, with populations of senescent cells shown to be highly functional and significantly contributing to the immune dysfunction seen with ageing.

Indeed, the age-associated loss of the costimulatory molecules CD27 and CD28 on CD4 and CD8 T cells is associated with reduced proliferative capacity, but also with gain of potent cytotoxic function (e.g. release of perforin, granzymes and IFNγ) mediated in part by TCR-independent activation of upregulated NK-like receptors, including NKG2D (79, 80). The late differentiated T_{EMRA} population (characterised by the re-expression of CD45RA) exhibit potent cytotoxicity and secretion of pro-inflammatory cytokines, but with limited proliferative capacity, which is mediated by inhibition of protein kinase p38 (81, 82). Late memory $IgD^{-}CD27^{-}$ B cell expansions are associated with increasing age (83), and although they exhibit reduced mitogen-induced replicative capacity in vitro

("senescence") compared to naïve B cells, they are avid producers of pro-inflammatory cytokines (e.g. TNFα and IL-6) and microRNAs (83). Such pro-inflammatory cytokine production has been described as the "senescence-associated secretory phenotype" (SASP). SASP contributes to the low-grade chronic inflammation seen with older age, characterised by elevated levels of circulating CRP, IL-6 and TNFα (78).

In addition to alterations in T cell responsiveness and function, the quality of antibody responses also declines with age. This results from a combination of defective B cell AID induction (a key enzyme involved in class switch recombination and somatic hypermutation) and reduced $CD4^+$ T cell functionality after TCR ligation (due to reduced expression of miR-181a), with resultant reduction in B cell help (78).

Alterations in adaptive immune regulation also occur with ageing. Although differentiation of regulatory T cells in the thymus and periphery declines with age, mature T_{regs} accumulate in the blood and secondary lymphoid organs through, as yet, incompletely understood mechanisms (84). Effector function of mature T_{regs} may cause excessive inhibition of otherwise useful immune responses, leading to poor pathogen clearance and increased susceptibility to infection. A reduced frequency and function (namely IL-10 secretion) of $CD24^{high}CD38^{high}$ regulatory B cells has also been reported in older adults (85). This is associated with increased circulating autoantibody, suggesting a mechanism for reduced immune tolerance seen with ageing (85).

1.8.3 The role of cytomegalovirus in shaping the adaptive immune system

Expansions of late differentiated T cells reported with older age have also been associated with chronic antigenic stimulation e.g. from persistent viral infections, calling into question their description as "age-related defects". Cytomegalovirus (CMV) is increasingly recognised as having a potent role in modulating the "immune ageing" phenotype (86).

A dsDNA betaherpesvirus, CMV establishes life-long persistent infection in humans after primary infection (often occurring in childhood) and periodically reactivates in response to stressors such as inflammation, infection or immunosuppression (87). These reactivations are usually subclinical and do not cause overt disease in the (otherwise) immunocompetent host. However, such chronic exposure to CMV antigens drives the expansion of CMV-specific memory T lymphocytes, particularly of the $CD28^{null}$ and T_{EMRA} phenotype (88, 89). Latent CMV infection has been associated with a number of other adverse outcomes in humans, including increased morbidity and mortality from cardiovascular disease (90). Indeed, $CD28^{null}$ $CD4^{+}$ T cells have potential for vascular endothelial toxicity (through expression of perforin/granzymes and endothelial homing markers CX3CR1, CD49d and C11b) and their expansions are independently associated with increased arterial stiffness (91, 92).

The effect of latent CMV infection on B lymphocytes is less clear, with limited and variable literature on this subject to date (93, 94). However, the magnitude of the humoral response to CMV mirrors the T cell effects seen. Indeed, serum CMV-specific IgG levels

are considered a reasonable surrogate for the magnitude of CMV-associated T cell effects (95).

Interestingly, the presence of latent CMV infection has also previously been associated with impaired responses to T-dependent vaccines in older adults (96) and a recent clinical trial demonstrated that suppression of CMV reactivation with valacyclovir could improve humoral responses to PCV13 in patients with vasculitis (97).

Several elegant studies have identified enhanced p38 MAPK signalling in T_{EMRA} populations as the mechanism for proliferative senescence and a potential therapeutic target to improve cellular function (81, 82, 98). However, the presence of highly functional and cytotoxic CMV-specific cells that are capable of controlling infection, but do not vigorously proliferate could actually be seen as a beneficial immune adaptation, rather than a defect (78), calling into question the potential therapeutic benefits of its reversal.

Another important factor associated with increasing susceptibility to infection and poorer vaccine responses in older age is declining health status and the development of a number of chronic conditions, including chronic kidney disease, which I will describe in the next section of this Chapter.

1.9 Chronic kidney disease (CKD)

Chronic kidney disease (CKD) describes the end-point of a number of heterogeneous disease processes that irreversibly alter kidney structure and function over a period of months or years. Current international guidelines define CKD as the presence of reduced

kidney function (a glomerular filtration rate (GFR) of less than 60ml/min/1.73m^2) or at least one marker of kidney damage (including proteinuria or other urinary sediment abnormality, histological or structural abnormalities) for a minimum of 3 months (99).

1.9.1 Epidemiology of CKD

CKD is an important global clinical problem. Although worldwide estimates of CKD prevalence and incidence are limited by heterogeneity in populations studied and CKD definition, it is consistently reported to affect approximately one in 10 individuals in high-income countries e.g. UK and USA (100, 101). The prevalence of CKD varies within countries by social class and ethnicity. Individuals within the higher socioeconomic quartile have a 60% lower risk of progressive CKD than those in the lowest quartile (100). Hispanics in the USA and Black and Asian individuals in the UK have a significantly greater risk of developing CKD, which is not fully explained by differences in socioeconomic status (100). CKD prevalence increases markedly with advancing age, affecting more than 30% of individuals aged 75 years and over (102). As such, the prevalence of CKD is predicted to increase further with the expansion of the ageing population.

CKD confers a significantly increased risk of all-cause mortality. Indeed, Global Burden of Disease Study estimates rank CKD as the 13th leading cause of death worldwide (103). CKD is also associated with significant morbidity (estimated age-adjusted global disability-adjusted life years (DALY) rate of 497.3 per 100 000 people in 2013 (103)) and associated economic burden, with CKD management accounting for approximately 1.3% of the 2009-2010 UK National Health Service (NHS) budget (£1.45 billion) (104).

Although global age-adjusted mortality and DALY rates due to non-communicable diseases in have been largely declining, this has not been the case with CKD. Infact, CKD-related mortality rates have increased by approximately a third and DALY rates by 12% from 1990 to 2013 (103).

1.9.2 Measuring kidney function

Glomerular filtration is the passive process of creating an ultrafiltrate from blood as it passes through the glomerular capillary network (102). It cannot be measured directly, but can be assessed by measuring clearance of exogenous compounds or estimated from serum levels of endogenous filtration markers such as creatinine (a low molecular weight metabolite) or the serum protein, cystatin C (102). Estimated GFR (eGFR) is the most widely used in clinical practice and is calculated using regression equations. GFR varies with body mass, dietary protein intake, age, gender and ethnicity, therefore GFR estimation equations include parameters to account for this and also standardise GFR to average body surface area (BSA) – $1.73m^2$ (102, 105). Two creatinine equations are currently used in clinical practice in the UK – the MDRD Study equation (106) (updated for use with standardised serum creatinine in 2006) and CKD-EPI creatinine equation (2009, (107)) – Table 1-11. The CKD-EPI creatinine equation includes the same four variables as the MDRD Study equation, but uses a 2-slope linear spline to model the relationship between serum creatinine and eGFR. The CKD-EPI creatinine equation is more accurate than the MDRD Study equation at higher levels of eGFR ($>60ml/min/1.73m^2$), resulting in lower rates of false-positive diagnoses of CKD (107).

Current guidelines advocate the use of CKD-EPI equation over that of the MDRD study (99).

Table 1-11 Regression equations for estimation of GFR using serum creatinine.

MDRD: Modification of Diet in Renal Disease study; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; SCr – serum creatinine in mg/dl. Compiled from references (106) and (107).

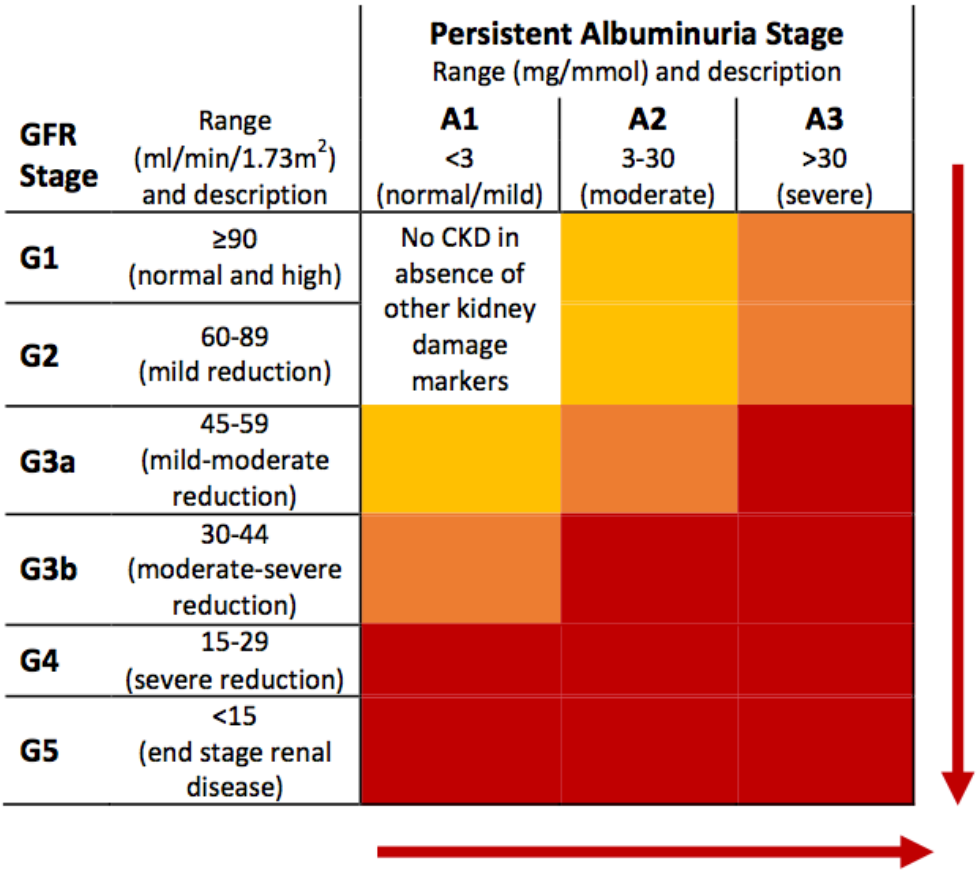
MDRD (2006)	175 x $SCr^{-1.154}$ x $age^{-0.203}$ x 1.212 [if Black] x 0.742 [if female]
CKD-EPI (2009)	141 x $\min(SCr/[0.9 \text{ if male}, 0.7 \text{ if female}], 1)^{[-0.411 \text{ if male}, -0.329 \text{ if female}]}$ x $\max(SCr/[0.9 \text{ if male}, 0.7 \text{ if female}], 1)^{-1.209}$ x 0.993^{Age} x 1.159 [if Black] x 1.018 [if female]

1.9.3 Classification of CKD severity

CKD severity is classified by the degree of renal impairment (GFR) and proteinuria as a marker of kidney damage (urinary albumin/creatinine ratio, ACR), as shown in Table 1-12. When kidney function drops below 15ml/min/1.73m^2 (CKD category G5), it is not sufficient to sustain physiological processes in the long term and is described as end stage renal disease (ESRD) (100). The risk of adverse patient outcomes increases with diminishing renal function and worsening proteinuria.

Table 1-12 Classification of CKD using GFR and ACR categories.

GFR: glomerular filtration rate; ACR: albumin/creatinine ratio; CKD: chronic kidney disease. Red arrows denote increasing risk of adverse outcomes. Adapted from reference (99) under the Creative Commons License.



1.9.4 Causes of CKD

Causes of CKD include, but are not limited to, those listed in Table 1-13. Although the causes of CKD vary globally, diabetes mellitus (DM) and hypertension (HTN) are the main causes of CKD in high and middle-income countries (100). CKD prevalence increases with age, mirroring the age-associated increasing prevalence of DM and HTN.

Table 1-13 Causes of chronic kidney disease (CKD) – a summary.

Ig: immunoglobulin; HIV: human immunodeficiency virus; NSAIDs: non-steroidal anti-inflammatory drugs. Compiled from reference (100).

Causes of CKD	Example
Systemic	Diabetes Mellitus
	Hypertension
	Renal vascular disease (atherosclerosis)
Inflammatory	IgA nephropathy
	Glomerulonephritis
Structural	Congenital single kidney
	Urinary tract abnormalities
Genetic	Polycystic kidney disease
Infection-related	HIV
	Pyelonephritis
Drug-induced	Lithium
	NSAIDs

HTN is common in CKD with a prevalence of over 80% in adults with non-dialysis CKD (GFR 15-60ml/min/1.73m^2) (108) compared with just over a quarter of the general population (109). The pathophysiological relationship between hypertension (HTN) and CKD is bidirectional, with declining kidney function typically associated with a rise in blood pressure (BP) and chronically elevated BP associated with an increased risk of CKD progression (110). HTN in CKD arises from a combination of factors including retention of sodium and water associated with reduced GFR, inappropriate activation of the renin-angiotensin-aldosterone system (RAAS) in the presence of a high sodium load, sympathetic nervous system activation and vascular endothelial dysfunction (111, 112). Treatment of HTN in CKD is often challenging and frequently requires the use of multiple anti-hypertensive therapies, including diuretics and RAAS inhibitors (113).

Diabetes mellitus (DM), one of the main causes of CKD is also a major cardiovascular risk factor. Although diabetic nephropathy accounts for approximately a third of ESRD

requiring RRT (114), the prevalence of co-existing DM in CKD is often higher (115). The presence of DM in CKD is associated with worse patient outcomes, including increased risk of progression to ESRD and greater all-cause mortality (116).

Although initial mechanisms of kidney damage differ, most chronic kidney diseases eventually result in renal fibrosis – the final stage of unsuccessful wound-healing, characterised histopathologically by glomerulosclerosis, tubular atrophy and interstitial fibrosis (100). The main goals of CKD treatment are to reverse potentially reversible pathologies (e.g. inflammation in glomerulonephritis), prevent deterioration of renal function (this includes the control of associated comorbidities e.g. HTN, DM), manage complications and, as disease progresses, prepare for RRT.

1.9.5 Clinical manifestations of CKD

Individuals with CKD are frequently asymptomatic until renal function is severely impaired and, as such, are often identified from routine screening tests. It is therefore difficult to estimate the true duration of CKD for most individuals.

As kidney function declines, a number of substances, collectively termed uraemic retention solutes, accumulate and exert effects on nearly all organs and body systems (summarised in Table 1-14). Solutes that exert adverse biological effects are referred to as uraemic toxins and their control is a major focus of current research as a therapy to ameliorate CKD progression and/or symptoms. However, accumulation of uraemic toxins is not always predictable, with marked variation between individuals in their biological effects at different levels of GFR (100).

Table 1-14 Main clinical manifestations of chronic kidney disease.

USS: ultrasound scan. Compiled from reference (100).

Organ System	Symptoms/Signs
General	Pallor Itch Muscle cramps – worse at night
Cardiovascular/Respiratory	Shortness of breath – fluid overload, anaemia, IHD Peripheral oedema Hypertension
Gastrointestinal	Anorexia Taste disturbance Nausea/vomiting
Urinary tract	Polyuria Oliguria Nocturia Haematuria USS – small kidneys, clubbed calyces/cortical scarring (chronic infection with reflux; or ischaemia), enlarged cystic kidneys
Central nervous system	Cognitive impairment

1.9.6 Complications of CKD

Table 1-15 summarises the main complications associated with CKD, several of which will be discussed in more detail below.

Table 1-15 Main complications of chronic kidney disease (CKD).

ESRD: end-stage renal disease; K^+: potassium; Na^+: sodium. Compiled from reference (100).

Complications of CKD
Progression to ESRD – requiring renal replacement therapy (RRT)
Electrolyte disturbance (e.g. K^+, Na^+)
Metabolic acidosis
Anaemia
Bone mineral disorder
Cardiovascular disease
Immune dysfunction
Protein-energy malnutrition
Malignancy

1.9.6.1 Progression to ESRD

CKD progression is defined as a sustained drop in eGFR of 25% or more and a change in GFR category over 1 year, and/or a sustained drop in eGFR of >15ml/min over 1 year (113), and results from the progressive loss of functional nephrons. Risk factors for CKD progression include male gender, Black/Asian ethnicity, proteinuria and the presence of DM, HTN, cardiovascular disease and anaemia (117-119). Although prevalence of CKD increases with age, older adults with CKD generally show slower rates of progression than younger populations (120).

ESRD is associated with the greatest risk of adverse outcomes and carries significantly increased risk of mortality compared to the general population (100, 121). Indeed, 1-year

mortality in patients with ESRD is typically greater than 10%, with cardiovascular disease and infection reported as the most common causes of death (114).

Therapeutic options for individuals with ESRD are renal replacement therapy (RRT - dialysis or kidney transplantation) or conservative care with symptom management (100). The UK prevalence of RRT in 2016 was 962 per million population – an increase of approximately 3% from the previous year, with a median patient age of 59 years (118). Kidney transplantation accounted for just over half of prevalent RRT, with haemodialysis accounting for 40% and peritoneal dialysis for 6% (118).

1.9.6.2 Anaemia

Typically normocytic and normochromic, anaemia is common in CKD, with prevalence increasing as kidney function declines. It arises from a combination of factors, including erythropoietin (EPO) deficiency through loss of cortical peritubular fibroblasts (the chief producers of EPO (122)), uraemia-associated reduced red cell survival and deficiency of haematinics such as iron and folate (100). Indeed, CKD is associated with excess circulating levels of hepcidin (123, 124), which impairs absorption of dietary iron.

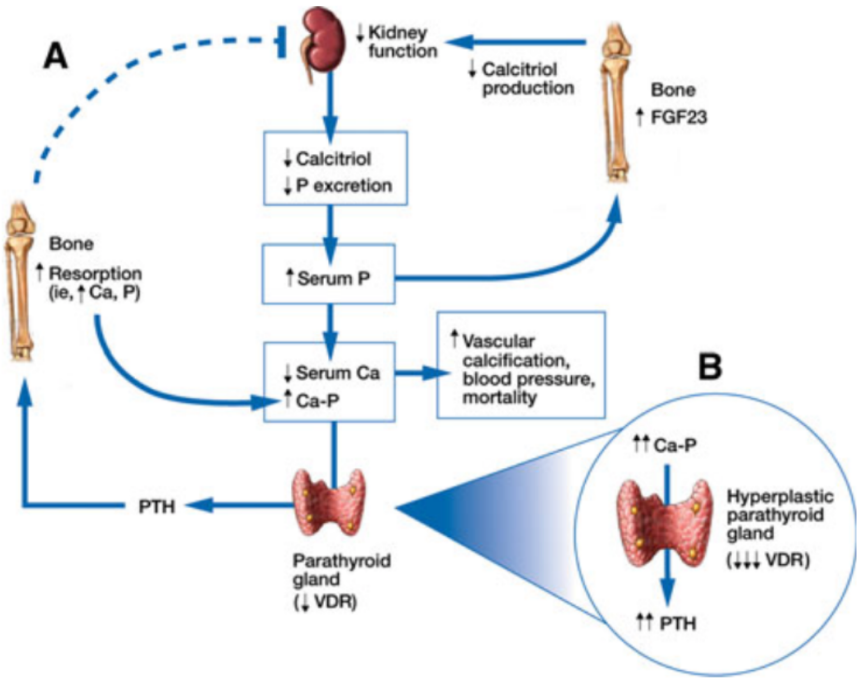
CKD-associated anaemia is an independent predictor of mortality and is associated with significant patient morbidity including reduced quality of life, more frequent hospital admissions and increased incidence of cardiac disease (125, 126). Treatment of CKD-associated anaemia was revolutionised with the advent of recombinant human EPO (rhEPO) and its synthetic derivatives (collectively termed erythropoiesis-stimulating agents, ESAs) (127).

1.9.6.3 Disordered bone mineral metabolism

The kidney plays a central role in maintaining calcium and phosphate homeostasis through interaction with parathyroid hormone (PTH), vitamin D and fibroblast growth factor 23 (FGF23). CKD-associated alterations in bone mineral metabolism are summarised in Figure 1-13.

Figure 1-13 Summary of alterations in bone mineral metabolism in chronic kidney disease (CKD).

A – CKD is associated with vitamin D deficiency and retention of phosphate (P). High phosphate stimulates secretion of bone-derived fibroblast growth factor (FGF) 23, which suppresses activation of vitamin D to calcitriol. Elevated calcium-phosphate product (Ca-P) stimulates secretion of parathyroid hormone (PTH), which increases resorption of bone. **B** – prolonged parathyroid gland stimulation results in hyperplasia and secondary/tertiary hyperparathyroidism. Ca: calcium; VDR: vitamin D receptor. Reproduced from reference (128) with permission from Springer Nature Limited.



Disordered bone mineral metabolism in CKD results in abnormalities in bone mineralisation, turnover and strength, which can present clinically as increased bone fragility, bone pain and/or extra-skeletal calcification (100).

1.9.6.4 Cardiovascular disease

Cardiovascular disease (CVD) is the most common cause of death in CKD, accounting for 1 in 4 deaths in prevalent RRT patients with ESRD in the UK (114). The risk of CVD-associated mortality increases in a stepwise manner with worsening CKD severity, independent of traditional CVD risk factors (129). Increasing albuminuria is also independently associated with increased CVD mortality risk, without a threshold effect, suggesting that even small increases in ACR are clinically significant (129).

The main early CKD-associated cardiovascular effects are left ventricular structural and functional abnormalities, together with arteriosclerosis (or vascular stiffening) (130). CKD is also associated with accelerated atherosclerosis (130), increased vascular calcification (131) and reduced coronary reserve and capillary density (132).

Increased CVD risk can be partly explained by the higher prevalence of several major traditional CVD risk factors in CKD populations, including HTN and DM (133). CKD is also independently associated with dyslipidaemia (111, 134) and “obese sarcopenia” (where a relative increase of adipose tissue mass is seen with declining lean tissue mass (135)).

However, CKD remains a strong independent risk factor for CVD mortality, even when traditional risk factors are taken into account (136). CKD-associated chronic low-grade

inflammation, increased oxidative stress and endothelial dysfunction are proposed explanatory mechanisms for this.

1.9.6.5 Chronic inflammation, increased oxidative stress and endothelial dysfunction in CKD

Albuminuria and reductions in GFR are associated with higher circulating biomarkers of inflammation, including C-reactive protein (CRP), tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) (137). Chronic low-grade inflammation is associated with worse outcomes in CKD, including progression to ESRD, the incidence of protein-energy malnutrition, anaemia and dysregulated bone mineral metabolism, together with cardiovascular and all-cause mortality (138, 139).

Several mechanisms have been proposed to cause this chronic inflammatory state. For example, retention of uraemic solutes including advanced glycation end (AGE) products can induce inflammation in target cells (e.g. vascular endothelial/smooth muscle cells) through direct and indirect activation of NF-κB and subsequent formation of pro-inflammatory cytokines (140). In addition to reduced renal clearance of reactive oxygen species (ROS), reductions in antioxidant factors such as superoxide dismutase and catalase (141) have also been reported. Endogenous production of ROS may also be increased in CKD through increased NADPH oxidase activity (141).

Increased circulating levels of endotoxins, likely due to translocation of gut bacteria as a result of increased gut permeability, have been observed in ESRD patients and correlate with conventional inflammatory markers (142).

CKD-associated “obese sarcopenia” has also increasingly been shown to contribute to the inflammatory milieu via adipokines such as leptin, circulating levels of which are elevated in ESRD (143).

Oxidative stress and chronic inflammation, together with retention of the nitric oxide synthesis inhibitor asymmetric dimethylarginine (ADMA), result in endothelial dysfunction in CKD. Inflammation, oxidative stress and endothelial dysfunction are key players in the accelerated atherosclerosis seen in CKD (141) and are also associated with non-atherosclerotic heart disease in non-dialysis CKD (144).

The chronic inflammatory state associated with CKD has been likened to that seen in chronological ageing, suggesting that renal impairment may accelerate progression of “normal” ageing processes.

1.10 The burden of infection in CKD

Patients with CKD have a greater susceptibility to infections and associated morbidity and mortality (121, 145). This was first observed as a marked excess incidence of infection-related death in patients with ESRD. Indeed, compared to the general population, ESRD treated with haemodialysis confers a 10-fold increased risk of death from pneumonia and 80 to 100-fold increased risk of death from sepsis (146, 147). One-year mortality in prevalent RRT patients with ESRD is typically greater than 10%, with infection accounting for almost 1 in 5 deaths, second only to cardiovascular disease (114).

The reported incidence of infections in patients with CKD is variable, but studies consistently show a graded increase in the risk of infection-related hospitalisation and

mortality both with reducing GFR (148-151) and increasing albuminuria, the impact of which is multiplicative (115). This is independent of potential confounders, such as age, gender, ethnicity, smoking/alcohol intake and prevalence of DM.

The magnitude of risk associated with renal impairment also varies between studies. For example, Dalrymple et al reported a 25% increase in all-cause infection-related hospitalisation in individuals with CKD stage G3b/4 (eGFR 15-44ml/min/1.73m^2) compared to those with an eGFR≥60 (149), whereas investigators from the Atherosclerosis Risk in Communities study (ARIC) reported at least a 2-fold increased risk (115).

Acute infections are associated with higher rates of hospitalisation and longer duration of stay in patients with CKD (152). The most common types of infections are respiratory and genitourinary (both accounting for approximately 20-25%) (115, 149) and recurrent infections are more common with worsening CKD severity (115).

Susceptibility to infection in CKD is not characterised by a predilection to any particular organism. Indeed, a study of bloodstream infection in a cohort of older adults with non-dialysis CKD showed an increased incidence of infection with a variety of Gram positive and Gram negative bacteria with worsening GFR (150).

Overall, infection-related hospitalisation episodes likely underestimate the true burden of infection in CKD. Factors associated with poor infection-related outcomes in patients with CKD include female gender (particularly in young haemodialysis patients), DM, CVD, hypoalbuminaemia, anaemia, and elevated inflammatory markers such as CRP (115, 147, 153). Interestingly, excess risk of infection associated with CKD may decline with increasing age (151), with differences in susceptibility to infection more pronounced in younger adults. It is important to note that, in addition to the excess risk of mortality, the

incidence of infection in patients with CKD is independently associated with a higher risk of cardiovascular events, including ischaemia and heart failure (153).

1.11 Vaccine responses in CKD

Amongst the reasons for the observed increased susceptibility to infection is that public health measures to reduce infection, such as vaccination, are not as effective in CKD as in the general population. This is best characterised for the hepatitis B virus (HBV) vaccine (154), where studies in patients with ESRD have consistently shown lower seroconversion rates (155-157) and faster decline in protective antibody titres than in healthy individuals (156, 158, 159). Increasing age (160-163), hypoalbuminaemia (164, 165) and obesity (166) have been associated with poorer HBV vaccination responses in patients with ESRD. Interestingly, better response to HBV vaccination in patients with ESRD have been associated with lower mortality rates (167).

Reflecting what is observed for the risk of infection, there is also a graded reduction in HBV vaccine responses with worsening renal impairment in non-dialysis CKD (168-171) and vaccination is currently recommended early in the disease to maximise humoral response (172). A number of other strategies have been employed in order to improve rates of seroconversion following HBV vaccination in CKD, including using higher vaccine doses (170) and increasing the number of doses given in a vaccination course (170, 173), but with variable effect (174, 175).

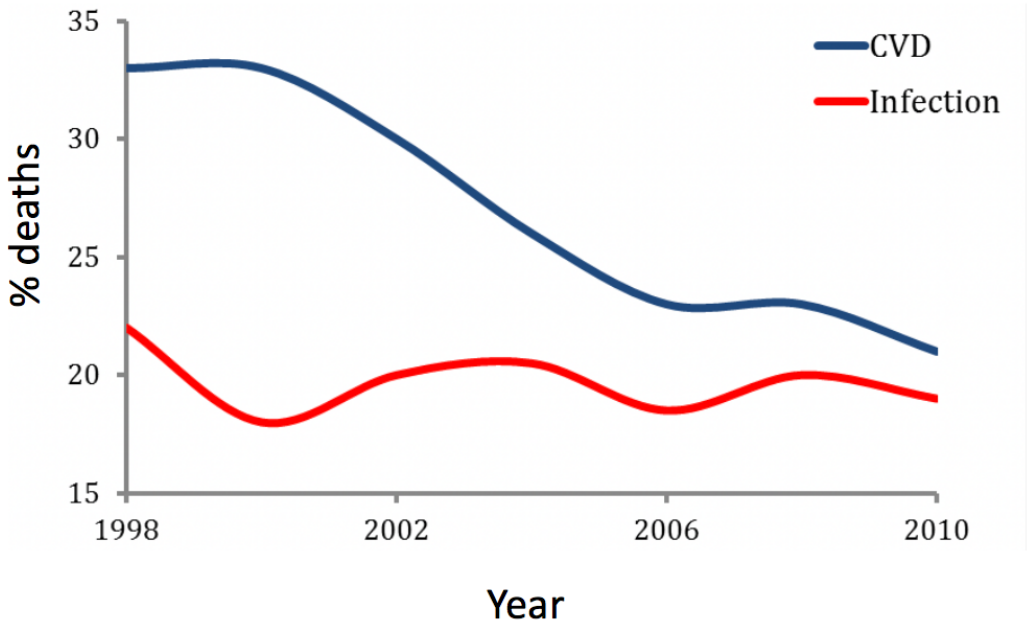
In addition to HBV, reduced responses in CKD/ESRD have also been reported for other TD vaccines e.g. influenza (176, 177) and also to TI vaccines including the pneumococcal polysaccharide vaccine (178, 179).

Interestingly, the magnitude of the humoral antigen-specific response to vaccination in CKD may depend on when the antigen was first encountered. Our group have recently shown relatively preserved humoral memory directed against historical vaccine antigens (DT/TT) and antigens that are repeatedly encountered throughout life (*Salmonella* lipopolysaccharide, CMV), which suggests maintenance of long-lived plasma cell niches established before the onset of CKD (180). This is also supported by the findings of Tsouchnikas et al (181) of a more rapid decline of anti-HBsAg antibodies generated following vaccination than those generated through natural infection in a cohort of patients with ESRD.

Collectively, the observations of increased susceptibility to infection and poorer vaccine responses in CKD have led to a dogma that these patients are immunodeficient and have a defective capacity to mount and maintain effective responses to antigens as compared to the general population. Although mortality from cardiovascular disease (CVD) in patients with severe CKD has markedly reduced over the past 20 years through innovations in diagnosis and patient management, mortality from infection remains largely unchanged (Figure 1-14) and the understanding of mechanisms behind poor immune responses in CKD remains incomplete.

Figure 1-14 Cause of death in prevalent renal replacement therapy cohorts by year.

Adapted from reference (145) under the Creative Commons Licence.



The next section of this chapter will summarise current understanding of the nature of immune defects in CKD, with a particular focus on chronic inflammation and dysfunction of neutrophils and lymphocytes.

1.12 The nature of immune dysfunction in CKD

The retention of various uraemic toxins has been proposed as a major driver of immune dysfunction in CKD (182). Host immune function in CKD has largely been studied in patients at the severe end of the disease spectrum (ESRD), who are receiving RRT, most commonly - haemodialysis. Immune function in less severe CKD has not yet been

comprehensively characterised, but, as renal disease is a continuum, it is potentially reasonable to suppose that alterations in immune function seen in ESRD start early in the course of CKD, just as other metabolic abnormalities associated with renal disease develop long before severe disease is established.

1.12.1 Neutrophils in CKD

A number of neutrophil functional defects have historically been identified in haemodialysis populations, including impaired phagocytosis (183, 184), reduced killing ability (185) and increased apoptosis (184). Neutrophils in patients with ESRD have also been shown to have features of spontaneous activation e.g. upregulation of TLR-2, TLR-4 and integrins, enhanced ROS production and degranulation (186), which may enhance ESRD-associated oxidative stress. However, some studies in ESRD have also shown reduced neutrophil ROS production (187).

Importantly, many of the neutrophil features seen in ESRD have been shown to be exacerbated following sessions of dialysis treatment (188, 189), particularly with the historical use of complement-activating cuprophane dialyser membranes (which also induced a potent transient neutropenia through neutrophil sequestration in the lung) (190-192), and with ESRD treatment complications such as iron overload (187) that are now infrequently seen in current practice. These observations call into question the validity of ascribing such neutrophil dysfunction to renal impairment *per se*, rather than its treatment.

A number of studies have examined neutrophils in non-dialysis CKD, with some findings directly conflicting the literature on ESRD. For example, ARIC study investigators showed a stepwise increase in circulating neutrophil numbers with reductions in eGFR

(193). Although neutrophil phagocytosis can be impaired in dialysis-treated patients, it has recently been shown to be comparable to controls in patients with less severe CKD (194). In this cohort, a stepwise increase in resting neutrophil oxidative burst was also seen with declining eGFR and responses to fMLP and *E coli* were reduced in patients receiving haemodialysis. However, in parallel to findings in ESRD populations, enhanced neutrophil apoptosis and increased expression of TLR2 and TLR4 with reducing eGFR have been reported in non-dialysis CKD (195-197).

1.12.2 Lymphocytes in CKD

Studies of lymphocyte populations in ESRD have reported reduced circulating total lymphocyte numbers (182, 186, 193). Lymphopenia, together with increased circulating neutrophils, results in an increased neutrophil/lymphocyte ratio (NLR). NLR may be a useful prognostic marker in ESRD, having been shown to predict cardiovascular and all-cause mortality in both peritoneal and haemodialysis populations (198-201), together with increased arterial stiffness (202, 203). NLR has also been proposed as a low-cost surrogate marker for CRP (204-207). In non-dialysis CKD, NLR has been consistently shown to be significantly higher than in age matched controls, with a step-wise increase with worsening CKD stage and a significant negative correlation with eGFR (208-210). As with dialysis populations, an elevated NLR has also been associated with greater incidence of cardiovascular events/mortality and all-cause mortality in non-dialysis CKD (209, 211).

1.12.2.1 T lymphocytes

Depletion of total circulating T cells has been reported in both ESRD and non-dialysis CKD populations (186, 212), together with variable reports of reductions in the CD4/8 ratio (212-214).

Increased T cell turnover and apoptosis have been observed in patients with ESRD, with resultant depletion of naïve (mirroring what is seen with immune ageing) and central memory T cells (214) that could reduce the capacity of the adaptive immune system to both recognise new antigens and respond to those previously encountered (186, 214, 215). Other T cell features consistent with immune ageing have been reported in CKD, including the expansion of terminally differentiated $CD28^{null}$ T cells, albeit in studies with variable controlling for the effects of latent CMV infection (216-219). Expansions of these $CD4^{+}CD28^{null}$ T cell populations have also been associated with increased CVD risk in CKD (92, 220). The senescence-associated secretory phenotype (SASP) of aged immune cells may also be responsible for the chronic inflammation seen in CKD (215).

T cell proliferation and functionality (characterised by activation-induced secretion of cytokines e.g. IL-2 and TNF-α (221, 222)) is reduced in ESRD (223). Indeed, Litjens et al demonstrated impaired HBsAg-specific T cell proliferation in samples taken from ESRD patients 2 weeks after HBV vaccination (which correlated with impaired serum antibody responses), together with impaired cytokine secretion (IL-2 and IFN-γ) (222). Polarisation of $CD4^{+}$ T cell effector responses towards the pro-inflammatory Th1 phenotype has been reported in ESRD as a putative driver of chronic inflammation (224) and a contributory mechanism to impairment of B cell function (through reduced Th2-mediated help) (225), but this is not a consistent finding (226, 227). Altered T_{reg} function in the context of

chronic immune over-activation is another hypothesis that has been proposed to explain the increased susceptibility to infections seen in CKD (221, 228). Both increased (221) and decreased T_{reg} numbers (229, 230) have been shown in dialysis-dependent CKD patients, together with increased T_{reg} apoptosis and impaired suppressive function (228, 231). This crucial inhibitory T cell population warrants further investigation, especially in non-dialysis CKD.

1.12.2.2 B lymphocytes

The effect of CKD/ESRD on B cell populations and their function is less well understood. Several groups have consistently reported B cell lymphopenia in ESRD (232-234) and non-dialysis CKD (235, 236). Reduction in circulating numbers of $CD27^{+}$ memory B cells has also been reported in paediatric patients with CKD (237).

Increased B cell apoptosis has been postulated as a mechanism for the observed contraction of the B cell pool in ESRD (232, 233, 238), but this is not a consistent finding (239). Another hypothesis for reduced circulating B cell numbers is uraemia-induced resistance to the action of B cell differentiation and survival factors e.g. BAFF (B cell-activating factor), that reduces the differentiation of transitional B cells into mature cells – Pahl et al recently identified reduced BAFF receptor expression on circulating transitional B cells in patients with ESRD, in the face of elevated circulating BAFF levels (239).

Although numerous studies have demonstrated impaired antibody responses to vaccination in ESRD/CKD, literature characterising the nature of B cell function *in vitro* or *in vivo* is limited. *In vitro* studies of B cell function in ESRD have generally shown reduced proliferation and impaired immunoglobulin secretion in response to a variety of antigens (238, 240, 241), but not consistently (242). However, these effects may be mediated by

dialysis treatment, rather than renal impairment itself (243). Interestingly, several groups have shown PTH-associated inhibition of B cell proliferative capacity (244-246), and improved *in vitro* B cell function with EPO treatment (243), suggesting other possible mechanisms for the observed CKD-associated immune dysfunction. The characterisation of B cell effector phenotypes during *in vitro* differentiation of B cells or the evaluation of *in vivo* generation of ASCs following antigen exposure in patients with CKD or ESRD has not, to my knowledge, previously been performed.

Overall, the immune phenotype reported in severe CKD is in keeping with a state of accelerated immune ageing. Indeed, immune cells in ESRD show greater telomere shortening compared to age-matched controls (247, 248) – a hallmark of replicative senescence. As such, CKD has been proposed as a phenotype of "unhealthy ageing". However, complete characterisation of immune system alterations associated with renal impairment, rather than its treatment (dialysis) and other potential immunomodulatory confounders e.g. CMV, remains incomplete. Interestingly, features described in ESRD suggest a dysregulation of immunity, rather than an abject failure to mount immune responses. Without improved understanding of where the defects lie in the development, maintenance, regulation and implementation of immune functions, potential practical approaches to reduce the burden of infection in CKD patients cannot be identified.

1.13 Aims of thesis

In this thesis I will describe the results of a prospective observational clinical study set up to test the hypothesis that older adults with mild-moderate renal impairment (CKD stage G3-4) have a dysregulated immune system compared to healthy age- and gender-matched

individuals. Two clinically recommended vaccinations (TIV and PPV23) were used as an *in vivo* antigen challenge and evaluation of multiple components/functions of both adaptive and innate immune systems was performed and compared between the groups.

In this study I took a non-biased approach to systematically characterise the immune "landscape" in CKD and examine both innate and adaptive immune cell phenotypes associated with dysfunctional responses to vaccines.

This study had 2 main aims:

Aim 1 (cross-sectional):

To investigate differences in the immune systems of patients with mild-moderate CKD and healthy individuals, in particular the relationship between innate and adaptive cell subsets and their function.

Aim 2 (observational with clinically recommended intervention):

To investigate differences in the response of adaptive immune system to external antigen challenge in patients with mild-moderate CKD compared to healthy individuals through real-time assessment of T-cell dependent pathways with seasonal influenza vaccination and T-cell independent pathways with pneumococcal polysaccharide vaccination.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical methods

The study described in this thesis was approved by the Edgbaston Research Ethics Committee (reference: 15/WM/0057) and the full protocol is presented in the Appendix.

2.1.1 Participants

Study participants were recruited to two groups – patients with non-dialysis CKD (n=36) and healthy controls (n=30). Recruitment and exclusion criteria are summarised in Table 2-1. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Table 2-1 Summary of SONIC study inclusion and exclusion criteria.

INCLUSION CRITERIA	
Any gender	
Aged 65 years and over	
EXCLUSION CRITERIA	
Aged < 65 years	
Does not have capacity as defined by Mental Capacity Act	
For control subjects:	eGFR <60ml/min **OR** any known renal disease
For CKD subjects:	eGFR ≤15ml/min or ≥60ml/min
Active infection and/or fever on baseline assessment	
Already received current year's seasonal influenza vaccine	
Received Pneumovax23© within last 5 years	
Any contraindications to influenza or pneumococcal vaccination	
Immunosuppressive conditions including, but not limited to:	• Malignancy within last 5 years (except non-melanoma skin cancer) • Solid organ or bone marrow transplant • Blood borne viral infections: HIV, hepatitis B and C • Autoimmune disease e.g. vasculitis, rheumatoid arthritis • Previous removal of spleen or asplenia from any other cause
Immunosuppressive medications, including, but not limited to:	• Systemic corticosteroids • Chemotherapy • Biological therapies within preceding 12 months

2.1.2 Recruitment

Study participants with CKD were recruited at the University Hospitals Birmingham NHS Foundation Trust (UHBFT) as summarised in Figure 2-1.

2.1.3 Intervention

Two seasonal vaccines – trivalent inactivated influenza and pneumococcal polysaccharide (Pneumovax 23©) – were used to examine immune responses to both T-dependent and T-independent antigens in the same individual. Vaccination in this study formed part of routine clinical care and the vaccines were not assessed for efficacy or tolerability.

The Pneumovax 23© vaccine (PPV23) manufactured by Merck (Philadelphia, USA) contained capsular polysaccharides for 23 *Streptococcus pneumoniae* serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

Composition of the seasonal trivalent influenza vaccine (TIV) varied from year to year based on recommendations provided annually by WHO on prevalent circulating strains (41). The TIV compositions for all 3 seasons of the SONIC study are shown in Table 2-2.

Both vaccines were administered intramuscularly into contralateral upper limbs at the recommended 0.5ml dose determined by the manufacturers.

Figure 2-1 Summary of recruitment methods for the SONIC study.

A - participants with CKD. Controls: **B** – relatives of patients with CKD, **C**- Birmingham 1000 Elders cohort.

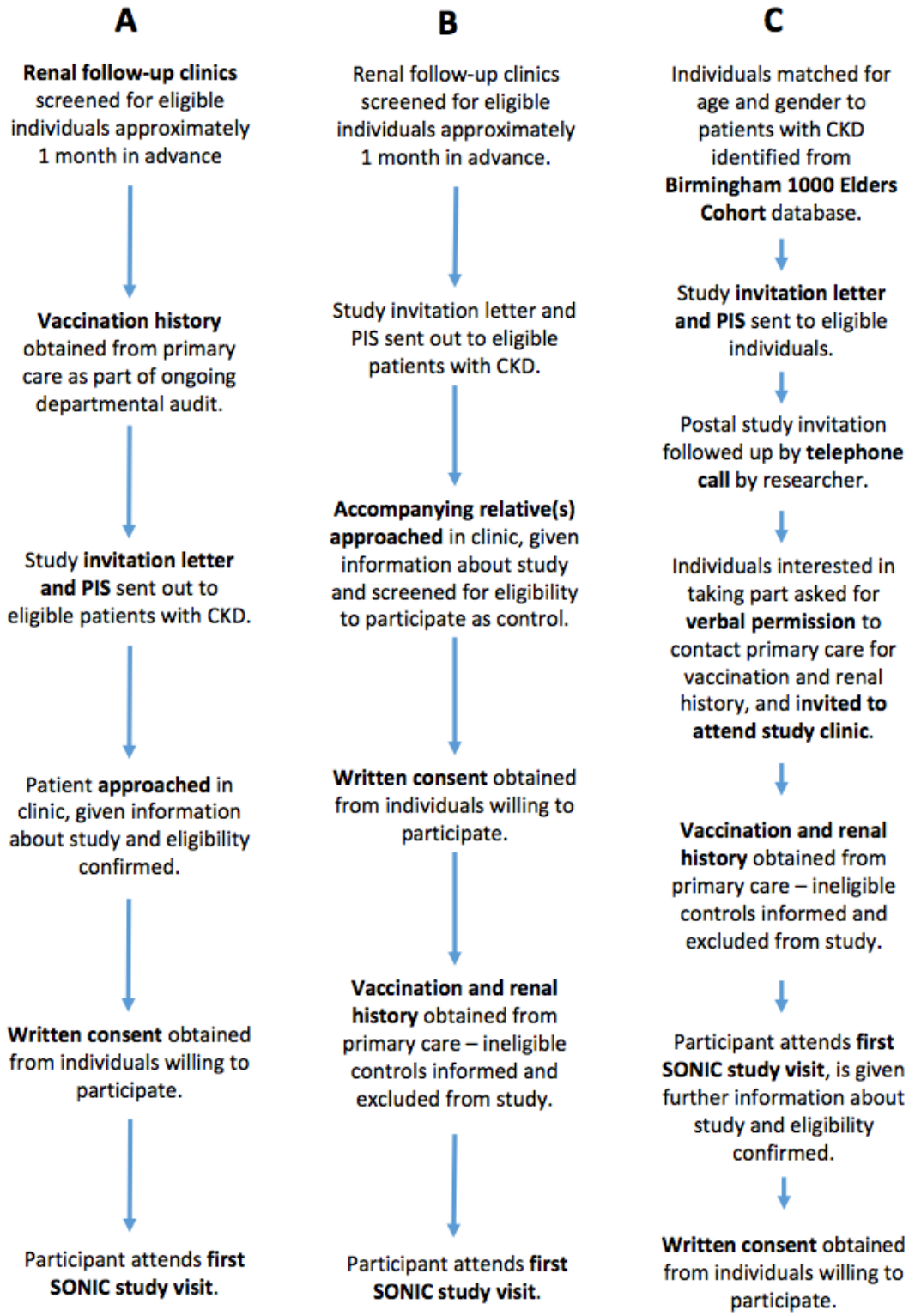


Table 2-2 Composition of seasonal trivalent influenza vaccines used in SONIC study.

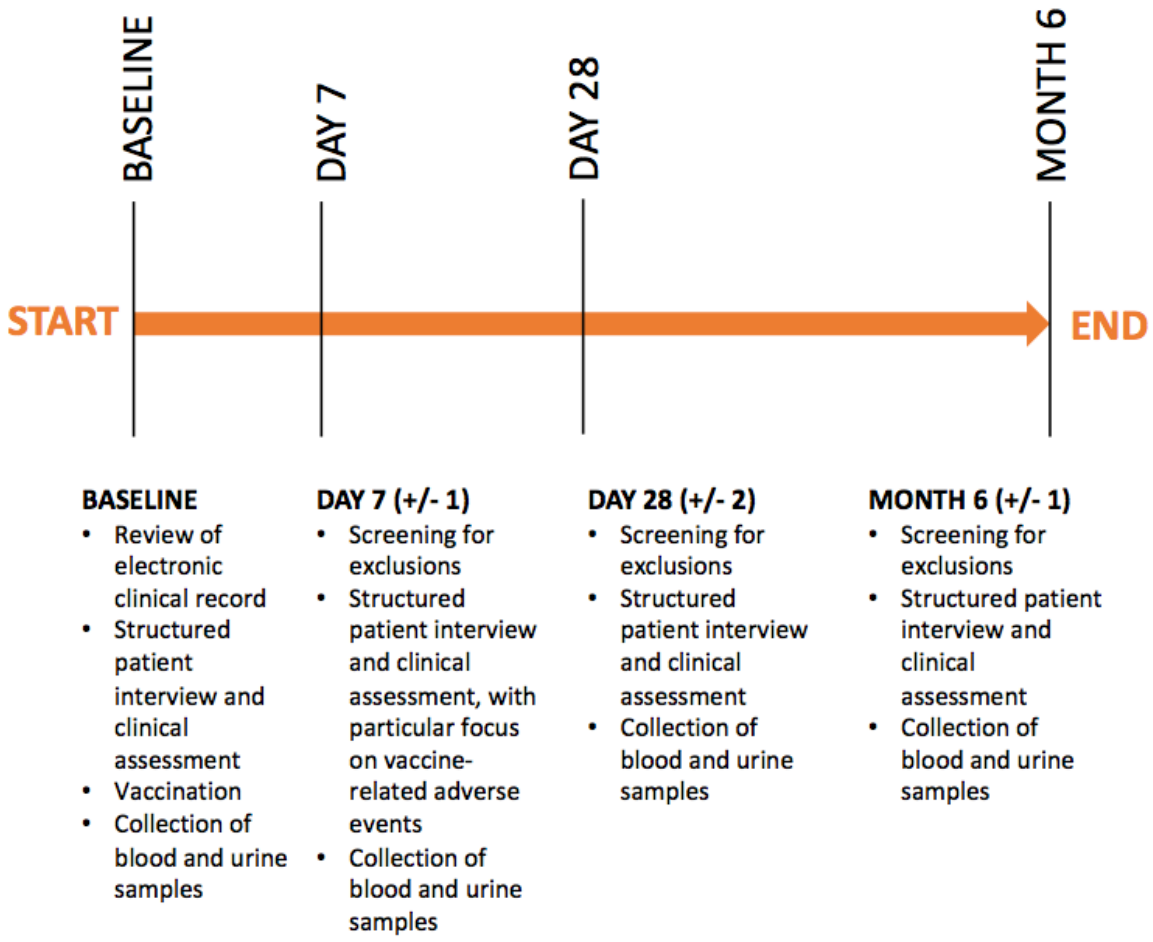
	2015-2016	2016-2017	2017-2018
Influenza A strains	A/California/7/2009 (H1N1)pdm09-like virus	A/California/7/2009 (H1N1)pdm09-like virus	A/Michigan/45/2015 (H1N1)pdm09-like virus
	A/Switzerland/9715293/2013 (H3N2)-like virus	A/Hong Kong/4801/2013 (H3N2)-like virus	A/Hong Kong/4801/2013 (H3N2)-like virus
Influenza B strains	B/Phuket/3073/2013-like virus	B/Brisbane/60/2008-like virus	B/Brisbane/60/2008-like virus

2.1.4 Schedule of visits

Participants were scheduled to attend a total of 4 study visits over 6 months – baseline (vaccination visit) and day 7, day 28 and month 6 after vaccination. Figure 2-2 summarises clinical activity at each study visit.

Demographics and baseline clinical information were sourced from electronic clinical records and patient interview at the baseline (vaccination) study visit. Vaccination history was obtained from primary care records requested as part of screening for study eligibility. Body mass index (BMI) was initially sourced from electronic records (within 6 months of baseline visit date), but this approach yielded a large amount of missing data for the first two vaccination seasons, so it was then recorded *de novo* at baseline for all study participants in the 2017-18 vaccination season.

Figure 2-2 Summary of SONIC study visit schedule and clinical activity at each visit.



Structured patient interview and examination at day 7 identified vaccine-related adverse events. Patient interview and review of electronic health records identified medication changes and new health events, including incidence of infections. No clinical microbiology samples or data were collected as part of this study to confirm/type infections. As such, patient reporting of infections was subjective and liable to recall bias.

2.1.5 Study outcomes and sample size calculation

The primary and secondary outcomes of the SONIC study were to identify the proportion of patients with CKD and controls that:

1. achieved post-vaccination antibody titres above levels associated with protection from disease and/or an adequate response to TIV and PPV23;
2. maintained "protective" antibody titre at 6 months' post-vaccination.

As such, the calculation of sample size was based on previous literature on humoral responses to the two study vaccines. Literature on humoral responses to PPV23 only was used as influenza literature was too heterogeneous due to yearly variations in vaccine composition. Few studies have evaluated responses to PPV23 in older pre-dialysis CKD patients. I therefore used studies of vaccination responses in healthy elderly individuals (249, 250) to estimate sample size by extrapolating the proportional reduction in response seen in studies of dialysis and renal transplant patients (251-254). Concentrations of antibody to pneumococcal polysaccharide (PnPS) are, by convention, reported as geometric means. A sample of approximately 50 individuals per group (total sample size 100) was calculated to have 80% power to detect a 2-fold reduction in antibody titre difference in patients with CKD as measured using a logarithmic scale.

Evaluation of the relationship between CKD and immune cell phenotype/function at baseline and after vaccination, together with the incidence of infectious disease requiring hospitalisation, GP consultation and/or antibiotics over a period of 6 months, were investigatory outcomes of the study.

2.1.6 Statistics

Statistical analyses were performed using Prism (version 7, GraphPad, La Jolla, CA, USA), SPSS software (version 24, IBM, New York, USA) and Open Source Epidemiologic Statistics for Public Health (free open source online software for epidemiologic statistics, available at http://openepi.com). Continuous data were tested for Normality (using Shapiro-Wilk or Kolmogorov Smirnoff tests) and groups were compared using parametric (unpaired/paired t test, ANOVA) and non-parametric methods (Mann Whitney and Kruskall Wallis tests) as appropriate. Categorical data were compared using Fisher's exact or Chi square tests. Differences in incidence rates were assessed using the mid P exact test. Correlations between continuous data were assessed using Pearson's test (Normal/parametric data) and Spearman's rank test (non-parametric data). Multivariate analysis on continuous data (Normalised as appropriate) was performed using linear regression modelling. In all statistical analyses, $p < 0.05$ was considered statistically significant.

2.2 Laboratory methods

At each study visit, approximately 50ml of venous blood was drawn peripherally using the Vacutainer® Safety-Lok^{TM} system (Beckton Dickinson, UK) into sterile Vacutainer® tubes (Beckton Dickinson, UK) with additives as listed in Table 2-3. Mid-stream samples of urine were also collected at each study visit into sterile 25 ml universal tubes without boric acid.

Table 2-3 Summary of blood samples collected at each study visit and the additives in Vacutainer collection tubes used.

EDTA – ethylenediaminetetraacetic acid.

Volume of blood	Vacutainer tube colour/name	Additive
36ml	Green	Sodium heparin
6ml	Red - clot activator tube	Micronised silica particles
3.5ml	Gold - serum separator tube	Silica gel
4ml	Purple	EDTA

Samples obtained at each study visit were either processed in the research laboratory within an hour of collection or sent for analysis to the Clinical Haematology and Biochemistry Laboratories at UHBFT as described later in this chapter. All Clinical Haematology samples were analysed on the day of collection. A minimum 5ml aliquot of urine collected at baseline was sent to Clinical Biochemistry on the day of collection for analysis of albumin/creatinine ratio (ACR). Serum samples were initially analysed by Clinical Biochemistry in batches from frozen serum prepared in the research laboratory (first two vaccination seasons, 2015-2017) in order to reduce processing costs. However, this proved inefficient and just as costly as sending fresh samples on the day of collection, so I reverted to the latter for the final vaccination season (2017-2018).

2.2.1 Samples for assay development from healthy volunteers

Venous blood samples (maximum 50mls/donor) were collected from healthy young volunteers in the laboratory for the purposes of assay development. This was approved by the University of Birmingham Ethics committee (ref: ERN_13-0880) and written informed consent was obtained from all such donors in accordance with the Declaration of Helsinki.

2.2.2 Serum preparation

Whole blood was collected into clot activator (red) or serum separator tubes (gold), allowed to clot at room temperature for 1 hour (+/- 10 minutes) and then spun for 10 minutes at 3000rpm, 4°C in a 5804R eppendorf centrifuge (Hamburg, Germany).

2.2.3 Clinical laboratory measurements

Clinical haematology and biochemistry measurements were performed by the Clinical laboratories at UHBFT using automated systems - Haematology Analyser (Beckman Coulter, CA, USA) and Roche/Hitachi 702 Analyser (Basel, Switzerland). Turbidimetric assays to determine serum concentrations of total IgG/A/M and IgG subclasses 1-4 were performed by the Clinical Immunology Service at the University of Birmingham using the cobas® (Roche/Hitachi, Indianapolis, USA) and SPA_{PLUS} automated analyser systems (Binding Site, Birmingham, UK).

2.2.4 Highly sensitive C-reactive protein (hsCRP) measurement

Serum concentrations of C-reactive protein (CRP) were determined using a high sensitivity enzyme-linked immunosorbent assay (ELISA) from IBL International (Hamburg, Germany), as per manufacturer instructions. Briefly, microtiterstrips pre-coated with anti-CRP antibody were incubated with 100µl/well of diluted standard sera (1:100) and test sera (1:1000 in sample diluent). Each sample was run in duplicate. Covered microtiterstrips were incubated at room temperature (RT) for 30 minutes and then washed 3 times with kit phosphate-buffered Washing Solution at 200µl/well. In between washes, microtiterstrips were emptied by flicking contents into a waste container and blotting on absorbent paper. Kit Conjugate Solution (peroxidase-conjugated monoclonal anti-human CRP antibody) was then added at 100µl/well and incubated at RT for 30 minutes. Washing was repeated as described above prior to addition of kit Chromogen Solution (tetramethylbenzidine, TMB) at 100µl/well and incubation for 10 minutes at RT, protected from light. The reaction was stopped by addition of 50µl kit Stop Solution (0.5M sulphuric acid) to each well. Absorbance at 450nm was read using a microplate reader (BioTek® Synergy, NorthStar Scientific Ltd, UK) within 30 minutes of assay completion. The minimal detectable concentration of the assay was 0.02µg/ml.

2.2.5 Measurement of CMV-specific IgG

Serum CMV-specific IgG titre was determined using a semi-quantitative in-house ELISA as previously described (255). Briefly, diluted cell lysate purified from CMV-infected fibroblast cultures and uninfected cells were used to coat a 96 well plate. Samples were added in a 1:600 dilution together with standards for a calibration curve (pooled plasma

from three healthy CMV positive donors) and incubated for 30 minutes at RT. Secondary antibody (anti-human IgG-horseradish peroxidase, Southern Biotech, USA) was added after washing the plate with phosphate buffered saline (PBS)/0.05% Tween20, and incubated for a further 30 minutes at room temperature. The plate was developed with TMB solution and absorbance at 450nm was read using a microplate reader (BioTek® Synergy, NorthStar Scientific Ltd, UK). Optical density attributable to CMV-specific IgG was calculated by subtracting control lysate well values from the CMV lysate wells. A cut off of 10 arbitrary units was used to determine CMV seropositivity i.e. the presence of latent CMV infection.

2.2.6 Measurement of IgG to pneumococcal polysaccharides and historic vaccine antigens

This assay was performed by the Clinical Immunology Service at the University of Birmingham as previously described (256) using a mix of carboxylated fluorescent microbeads specific for distinct bead regions on a Luminex instrument (BioRad, CA, USA). The beads were conjugated to 1 of 14 different antigens (12 capsular polysaccharides: *Streptococcus pneumoniae* (Pn) serogroups 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F; 2 toxoids: Diphtheria and Tetanus; all sourced from ATCC, VA, USA). A dilution plate was made for each experiment prior to transfer to a 96-well filter microplate for incubation with the bead mix. Briefly, a series of 12 2-fold dilutions of 007-SF standard serum (Food & Drug Administration, USA) starting at 1:100 was prepared in standard dilution buffer (PBS, 1%BSA, 0.05%Tween 20 and 5µg/ml pneumococcal cell wall polysaccharide). Test serum was then added at a dilution of 1:100 in standard dilution

buffer with 5µg/ml of serotype 22F polysaccharide (added to absorb non-specific antibodies). Four controls were run with every plate – 2 negative (sample dilution buffer; antibody-depleted healthy donor serum) and 2 positive (1 healthy donor serum and 1 mix of 3 healthy donors). The reported intra- and inter-assay variability for this IgG assay are 8% and 31% respectively (256).

2.2.7 Influenza haemagglutination inhibition assay

The influenza haemagglutination inhibition (HAI) assay was performed by Rebecca Penn and Ruth Harvey from NIBSC laboratories (Potters Bar, UK) using a standard method as previously described (257). Briefly, sera were diluted 1:5 with receptor destroying enzyme (RDE), incubated overnight at 37°C and then heat inactivated at 56°C for 45 minutes. Sera were then added to a 96-well plate in a 2-fold dilution series in PBS with a final volume of 100µl per well (final dilutions 1:10 to 1:1280). Test influenza virus (pre-diluted in PBS) was added at 50µl per well (dilution factor was determined by dividing the virus haemagglutinin (HA) titre by 8) and the plate incubated at RT for 60 minutes. Turkey erythrocytes in a 0.7% suspension in PBS were then added at 50µl per well and the samples left at room temperature for at least 20 minutes, until cell settling could be seen. The plate was read by tilting and evaluating the last dilution of serum with complete "streaming" of non-agglutinated red cells. The reciprocal of this dilution was taken as the "HAI titre" of that serum sample. Samples that failed to agglutinate the first dilution (1:10) were assigned a HAI titre of 5. All test sera were tested twice against the relevant vaccine strains from each vaccine season. RDE treated ferret antisera were used as positive and negative controls.

2.2.8 Peripheral blood mononuclear cell (PBMC) isolation

Heparinised whole blood was diluted 1:1 with warm complete sterile Roswell Park Memorial Institute (RPMI) medium (RPMI 1640, 10% heat-inactivated fetal calf serum (HI FCS), 100U/ml penicillin, 100µg/ml streptomycin, all sourced from Sigma Aldrich, UK). PBMCs were isolated from diluted whole blood using gradient centrifugation with Ficoll Paque (GE Healthcare Life Sciences, IL, USA) and Leucosep tubes (Greiner-Bio-One International, Austria) as per manufacturer instructions, and washed twice with complete RPMI (50ml/Falcon tube, spun at 300g for 10 minutes at 4˚C) prior to counting using a haemocytometer. The average yield of PBMCs from donors was $1x10^6$ cells per 1 ml whole blood.

2.2.9 PBMC freezing and thawing

For storage, PBMCs were resuspended in ice cold freezing solution (90% HI FCS, 10% DMSO, both Sigma Aldrich, UK) immediately after isolation at $5\text{-}10x10^6$ cells/ml. Cells were transferred to cryovials and frozen initially at -80˚C using a Mr Frosty™ freezing container (ThermoFisher Scientific, MA, USA) or CoolCell™ (Biocision, CA, USA). Samples were then transferred to liquid nitrogen storage within 1-2 weeks for the first two years of the study. Unfortunately, PBMCs stored in liquid nitrogen had much greater variation in viability following thaw than cells frozen at -80˚C, so samples collected during the final year of the study were kept at -80˚C only.

When required, frozen PBMCs were removed from storage and quickly defrosted by immersion in a 37˚C water bath for less than 5 minutes. Cells were washed once in warmed complete RPMI (50ml/Falcon tube, spun at 300g for 10 minutes at RT), then

resuspended in 2ml complete RPMI prior to addition of benzonase (Merck Millipore, MA, USA) at 1µl per 10^6 cells frozen. Cells were then incubated for 1 hour at 37˚C and washed a further 2 times in warmed complete RPMI prior to use (for flow cytometry viability staining, the last wash was performed in warmed sterile PBS). Cells were counted after defrosting using a haemocytometer. Average yield was 50-70% of cells initially frozen down.

2.2.10 Neutrophil isolation from peripheral venous blood

Heparinised whole blood was first incubated with dextran (2% w/v, reconstituted in 0.9% saline; Amersham Biosciences, Uppsala, Sweden) at a dilution of 1:6 in a sterile 50ml Falcon™ tube (BD Biosciences, UK) at RT for 30 minutes to allow red cell sedimentation to occur. Leucocyte-rich plasma was then carefully layered using a fine tip Pasteur pipette onto a sterile Percoll® (Sigma Aldrich, UK) density gradient consisting of 5ml 56% and 2.5ml 80% Percoll® (diluted in 0.9% saline) in a sterile 15ml Falcon™ tube. Loaded gradients were spun at 1100rpm for 20 minutes with no brake at RT in an MR211*i* Jouan centrifuge (ThermoFisher Scientific, MA, USA). Neutrophils were then harvested from the 56-80% interface using a Pasteur pipette and transferred into a sterile 50ml Falcon™ tube. Cells were washed in RPMI-1640 medium supplemented with 1% penicillin/streptomycin (hereafter referred to as RPMI-PS) by spinning at 1600rpm, RT, for 10 minutes. After discarding supernatant, cells were resuspended in 1ml RPMI-PS prior to counting and determination of purity using an automated haematology analyser (Sysmex, UK). Cell populations containing ≥95% neutrophils were accepted for further experiments and diluted to a final working concentration in RPMI-PS.

2.2.11 Lymphocyte immunophenotyping

Flow cytometric phenotyping of T and B lymphocytes was performed using an LSR Fortessa instrument (BD Biosciences, UK) and results were analysed using FACSDiVa software version 8.0 (BD Biosciences, UK).

2.2.12 Viability staining

Viability staining was performed on all PBMC samples using an intracellular amine-reactive dye (LIVE/DEAD^{TM} near IR Fixable Dead Cell Kit, ThermoFisher Scientific, USA) prior to surface and/or intracellular staining. Briefly, viability dye was defrosted and reconstituted with 50µl anhydrous DMSO per vial, as per manufacturer instructions. PBMCs were prepared as described above (section 2.2.8), washed once in PBS immediately prior to staining and resuspended at 10^6 cells/ml. 1µl of dye was added to 1ml of cell suspension and incubated at RT for 30 minutes, protected from light. Cells were then washed with 2ml MACS buffer (PBS with 1% HI FCS and 2mM EDTA, all from Sigma Aldrich), spun at 300g for 5 minutes and resuspended in 100µl Brilliant^{TM} staining buffer (BD Biosciences, UK) for surface staining. Samples were excluded from final analysis if viability of the lymphocyte gate fell below 80%.

2.2.13 Surface staining

Titrated volumes of fluorochrome conjugated antibodies against cell surface markers were then added to the cell suspension in Brilliant^{TM} staining buffer (BD Biosciences, UK) and

incubated at 4°C for 30 minutes, protected from light. After staining, cells were washed once with MACS buffer, and either stained for intracellular markers or prepared for analysis (resuspended in 300µl MACS buffer per tube and kept at 4°C, protected from light) - usually performed within an hour. To correctly and consistently identify "positive" populations of cells, a fluorescence-minus-one (FMO) control, where the marker of interest was substituted in the panel with an equivalent concentration of an isotype control antibody, was performed for each experiment. Table 2-4 shows the T lymphocyte and Table 2-5 the B lymphocyte immunophenotyping panels used in this study. Freshly isolated PBMCs were used for Panels 1 and 2 as staining for these markers was suboptimal on frozen samples during assay development.

2.2.14 Intracellular staining for FoxP3

Intracellular staining for FoxP3 was performed after surface staining using the eBioscience FoxP3/Transcription Factor Staining Buffer Kit, as per manufacturer instructions. Briefly, the kit Fixation/Permeabilization Concentrate (containing <5% paraformaldehyde) was diluted 4-fold with the kit Fixation/Permeabilization Diluent and added at 1ml per tube to the PBMC pellet after surface stained cells were washed with MACS buffer. Cells were incubated at RT for 30 minutes, protected from light. 2ml of 1X Permeabilisation Buffer (10x concentrate in kit diluted with deionised water) was then added to each tube, vortexed and spun at 300g, RT for 5 minutes. Supernatant was discarded and the cell pellet resuspended in the residual 1X Permeabilisation Buffer volume (approximately 100µl). The PE-conjugated anti-human FoxP3 antibody (eBioscience, USA) was added at 5µl per tube (10^6 PBMCs), the mixture was vortexed

and incubated at 4°C for 30 minutes, protected from light. PBMCs were then washed twice – first with 1X Permeabilisation Buffer (2ml/tube), followed by MACS buffer (2ml/tube), before being resuspended in 300µl MACS buffer per tube, ready for analysis.

Table 2-4 T lymphocyte immunophenotyping flow cytometry panels used in this study.

Red colour denotes intracellular marker.

Panel 1: CD4 T cell phenotype					
Marker	**Clone**	**Fluorochrome**	**Isotype**	**Manufacturer**	**Volume/test**
CD3	UCTH1	Alexa Fluor 700	Mouse IgG_1, kappa	BD	3µl
CD4	SK3	BV711	Mouse IgG_1, kappa	BD	5µl
CD8	RPA-T8	BV510	Mouse IgG_1, kappa	BD	5µl
CD45RA	HI100	BV421	Mouse IgG_{2b}, kappa	BD	2.5µl
CCR7 (CD197)	150503	PE-CF594	Mouse IgG_{2a}	BD	5µl
CCR4 (CD194)	L291H4	PE-Cy7	Mouse IgG_1, kappa	Biolegend	2.5µl
CXCR5 (CD185)	J252D4	APC	Mouse IgG_1, kappa	Biolegend	2.5µl
CXCR3 (CD183)	G025H7	FITC	Mouse IgG_1, kappa	Biolegend	2.5µl
CCR6 (CD196)	G034E3	PerCP-Cy5.5	Mouse IgG_{2b}, kappa	Biolegend	2.5µl
Panel 2: Regulatory T cell phenotype					
Marker	**Clone**	**Fluorochrome**	**Isotype**	**Manufacturer**	**Volume/test**
CD3	UCTH1	Alexa Fluor 700	Mouse IgG_1, kappa	BD	3µl
CD4	RPA-T4	BV605	Mouse IgG_1, kappa	BD	5µl
CD25	M-A251	APC	Mouse IgG_1, kappa	BD	20µl
FoxP3	PCH101	PE	Rat IgG_{2a}, kappa	eBioscience	5µl
Panel 3: T cell naïve/memory phenotype					
Marker	**Clone**	**Fluorochrome**	**Isotype**	**Manufacturer**	**Volume/test**
CD3	UCTH1	Alexa Fluor 700	Mouse IgG_1, kappa	BD	3µl
CD4	SK3	BV711	Mouse IgG_1, kappa	BD	5µl
CD8	RPA-T8	BV510	Mouse IgG_1, kappa	BD	5µl
CD45RA	HI100	BV421	Mouse IgG_{2b}, kappa	BD	2.5µl
CCR7 (CD197)	150503	PE-CF594	Mouse IgG_{2a}	BD	5µl
CD27	M-T271	PE-Cy7	Mouse IgG_1, kappa	BD	2.5µl
CD28	CD28.2	APC	Mouse IgG_1, kappa	BD	10µl
CD57	HNK-1	PE	Mouse IgM, kappa	Biolegend	2.5µl
KLRG1	SA231A2	FITC	Mouse IgG_{2a}, kappa	Biolegend	2.5µl

Table 2-5 B lymphocyte immunophenotyping flow cytometry panels used in this study.

Panel 4: B cell naïve/memory and regulatory phenotypes					
Marker	Clone	Fluorochrome	Isotype	Manufacturer	Volume/test
CD40	5C3	Alexa Fluor 700	Mouse IgG_1, kappa	BD	5µl
IgD	IA6-2	PE-CF594	Mouse IgG_{2a}, kappa	BD	2.5µl
IgM	G20-127	BV510	Mouse IgG_1, kappa	BD	5µl
CD24	ML5	BV421	Mouse IgG_{2a}, kappa	BD	5µl
CD80	L307.4	PE-Cy7	Mouse IgG_1, kappa	BD	5µl
CD86	IT2.2	PE-Cy7	Mouse IgG_{2b}, kappa	Biolegend	2.5µl
CD38	HIT2	APC	Mouse IgG_1, kappa	BD	20µl
CD19	HIB19	BB515	Mouse IgG_1, kappa	BD	2.5µl
CD27	L128	BV650	Mouse IgG_1	BD	5µl
Panel 5: Plasma cells/blasts					
Marker	Clone	Fluorochrome	Isotype	Manufacturer	Volume/test
CD20	2H7	Alexa Fluor 700	Mouse IgG_{2b}, kappa	BD	2.5µl
CD38	HIT2	BV510	Mouse IgG_1, kappa	Biolegend	5µl
CD19	HIB19	BB515	Mouse IgG_1, kappa	BD	2.5µl
IgD	IA6-2	PE-CF594	Mouse IgG_{2a}, kappa	BD	2.5µl
CD27	L128	BV650	Mouse IgG_1	BD	5µl

2.2.15 Compensation method

For all immunophenotyping experiments, single stain compensation samples were used to determine spectral overlap using the automated compensation option in FACSDiVa software (BD Biosciences, UK). Single stain compensation samples were prepared using ArC™ Amine Reactive and AbC™ Total Antibody compensation beads (Invitrogen) as per manufacturer instructions with pre-titrated volumes of fluorochrome-conjugated antibodies and viability dye. Adequacy of automated compensation was checked manually following data acquisition on the flow cytometer.

2.2.16 Measurement of neutrophil phagocytosis and reactive oxygen species production

These functional parameters were assessed using Phagoburst™ and Phagotest™ assays (Glycotope GmbH, Berlin, Germany), as per manufacturer instructions.

The Phagoburst™ assay quantitatively determines neutrophil oxidative burst in response to a number of stimuli, including N-formylmethyonine-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA) and heat-killed opsonised *Escherichia coli*, though intracellular oxidation of dihydrorhodamine 123 (DHR-123) to its fluorescent product, rhodamine (258). Briefly, 100µl heparinised whole blood was transferred to a round bottom 5ml polystyrene tube (BD Biosciences, UK) - 1 tube was used for each of the following test conditions: wash buffer (negative control), fMLP (low biological control), PMA (positive control) and *E coli* (test condition). Samples were chilled on ice for a minimum of 10 minutes. Stimuli were added at 20µl per tube and samples were incubated for 10 minutes in a water bath at 37°C. The substrate (DHR-123) was then added at 20µl per tube and samples were incubated for a further 10 minutes at 37°C. Samples were incubated with 2ml 1x Fix/Lyse buffer for 20 minutes at RT, protected from light, and spun at 250g, 4°C for 5 minutes. Samples were then washed with 3ml/tube kit Wash Solution and spun at 250g, 4°C for 5 minutes, prior to addition of 200µl of DNA Staining Solution (propidium iodide) and incubation on ice for 10 minutes, protected from light.

The Phagotest™ assay evaluates leucocyte phagocytosis by identifying neutrophils that have ingested FITC-labelled inactivated opsonised *E. coli*. Briefly, 100µl heparinised whole blood was transferred to a round bottom 5ml polystyrene tube (BD Biosciences, UK) - 1 tube was used for the test condition and 1 for a negative control. Samples were

chilled on ice for a minimum of 10 minutes. FITC-labelled heat-killed opsonised *E. coli* were added at 20µl per tube. The negative control remained on ice, but the test sample was transferred to a water bath at 37°C and incubated for 10 minutes. Fluorescence of extracellular bacteria was quenched using 100µl/tube kit Quenching Solution (trypan blue) and samples were then washed with 3ml/tube kit Wash Solution and spun at 250g, 4°C for 5 minutes. Samples were then incubated with 2ml 1x Fix/Lyse buffer for 20 minutes at RT, protected from light, and spun at 250g, 4°C for 5 minutes. Another wash was performed prior to addition of 200µl of DNA Staining Solution (propidium iodide) and incubation on ice for 10 minutes, protected from light.

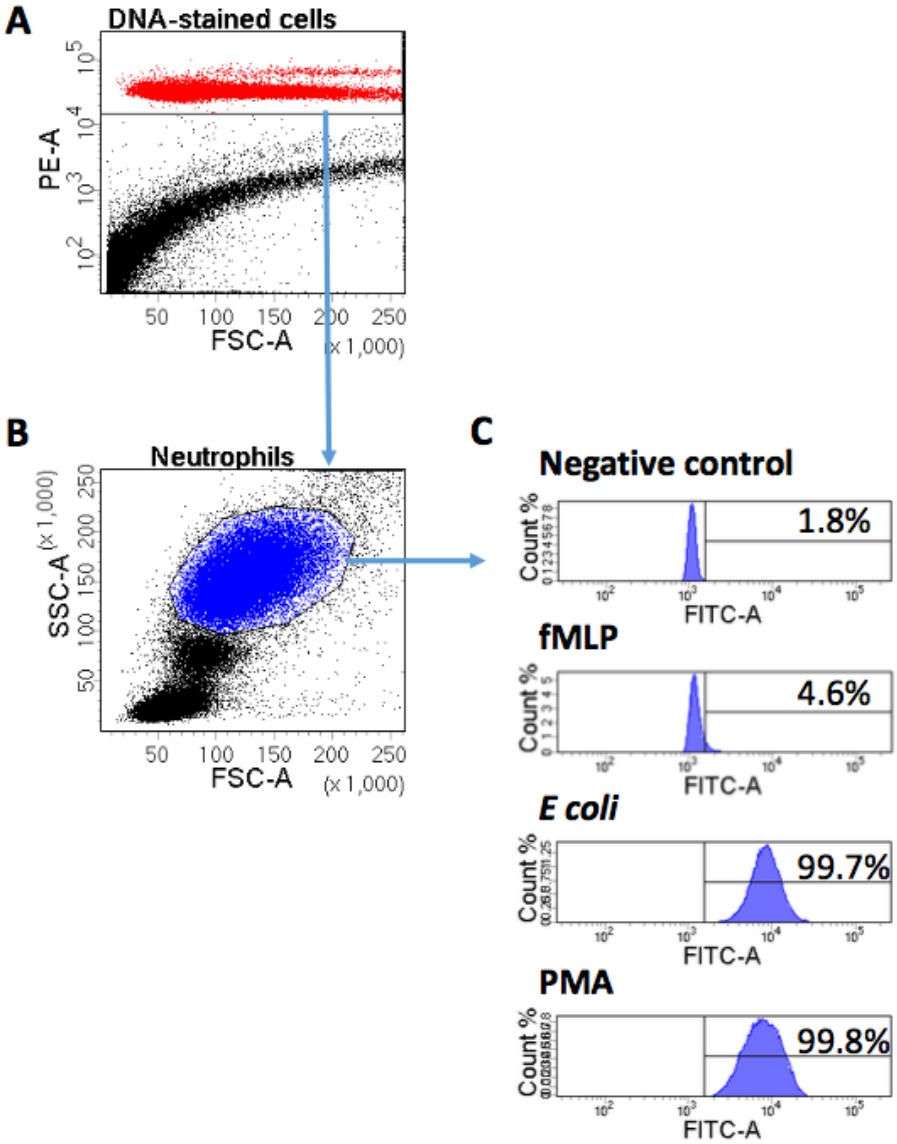
All samples were analysed using a BD LSR Fortessa instrument within 30 minutes of assay completion (10,000 - 15,000 leucocytes recorded per sample), using a similar gating strategy for both assays, as summarised in Figure 2-3. Bacterial aggregates and debris were excluded by gating on DNA-stained cells (Figure 2-3A) and neutrophils were identified by forward and side-scatter characteristics (Figure 2-3B). Fluorescence in the FITC channel was then recorded for each test condition (both % and mean fluorescence intensity (MFI) of the neutrophil gate), with the negative control used to determine cut-off for FITC "positivity" (Figure 2-3C).

The proportion of neutrophils defined as FITC positive by the above method were reported as "responding cells" in both assays. Phagocytic index (PI) was defined as the product of the phagocytosing neutrophil % and the FITC MFI of the neutrophil gate in the Phagotest™ assay, in line with previous studies (259). Oxidative burst capacity was defined as the FITC MFI of the neutrophil gate in the Phagoburst™ assay. The flow cytometer underwent repair after data acquisition for the 2015 cohort, hence FITC MFI in

data from this year is not directly comparable to subsequently acquired samples. In view of this, FITC MFI has been standardised across the entire study by dividing the MFI from stimulated samples by that of the negative control.

Figure 2-3 Example gating strategy for PhagoTest™ and PhagoBurst™ assays.

A – scatter plot demonstrating exclusion of bacterial aggregates/cell debris; **B** – scatter plot demonstrating selection of neutrophils; **C** – histogram of FITC fluorescence with representative data from study samples (PhagoBurst™ test conditions shown).



2.2.17 Quantification of neutrophil extracellular trap (NET) formation

Unprimed neutrophils suspended at $1x10^6$/ml in RPMI-PS were added to wells of a 96-well flat bottomed plate (BD Biosciences, UK) at 200µl ($2x10^5$ cells) per well. Cells were then stimulated using PMA (final concentration 25nM diluted in RPMI-PS; Sigma Aldrich) or RPMI-PS as a negative control in replicates of 4 for each condition and incubated for 3 hours at 37°C in a humidified 5% CO_2 atmosphere.

Post-incubation, samples were transferred to 500µl microcentrifuge tubes (Sarstedt, Germany) and pelleted at 2200g for 10 minutes at 4°C in a pre-cooled microcentrifuge (Eppendorf, Germany). From each tube, 100µl of cell-free supernatant was then transferred into wells of a flat bottomed 96-well black plate (Corning, New York, USA) prior to addition of the cell-impermeable DNA binding dye SYTOX® Green (Life Technologies, UK) at 1µM concentration. A standard curve of λ-DNA (ThermoFisher Scientific, UK) was run alongside each sample in a 1:2 dilution series (concentration range 0-1000ng/ml). The plate was incubated for 10 minutes at room temperature, protected from light, and extracellular DNA content was then quantified by measuring fluorescence using a BioTek® Synergy 2 fluorimetric plate reader (NorthStar Scientific Ltd, UK) with a filter setting of 485nm (excitation) and 528nm (emission). Concentration of extracellular DNA was determined from the DNA standard curve and the mean of 4 replicates was reported for each sample.

2.2.18 Visualisation of NET formation using fluorescent microscopy

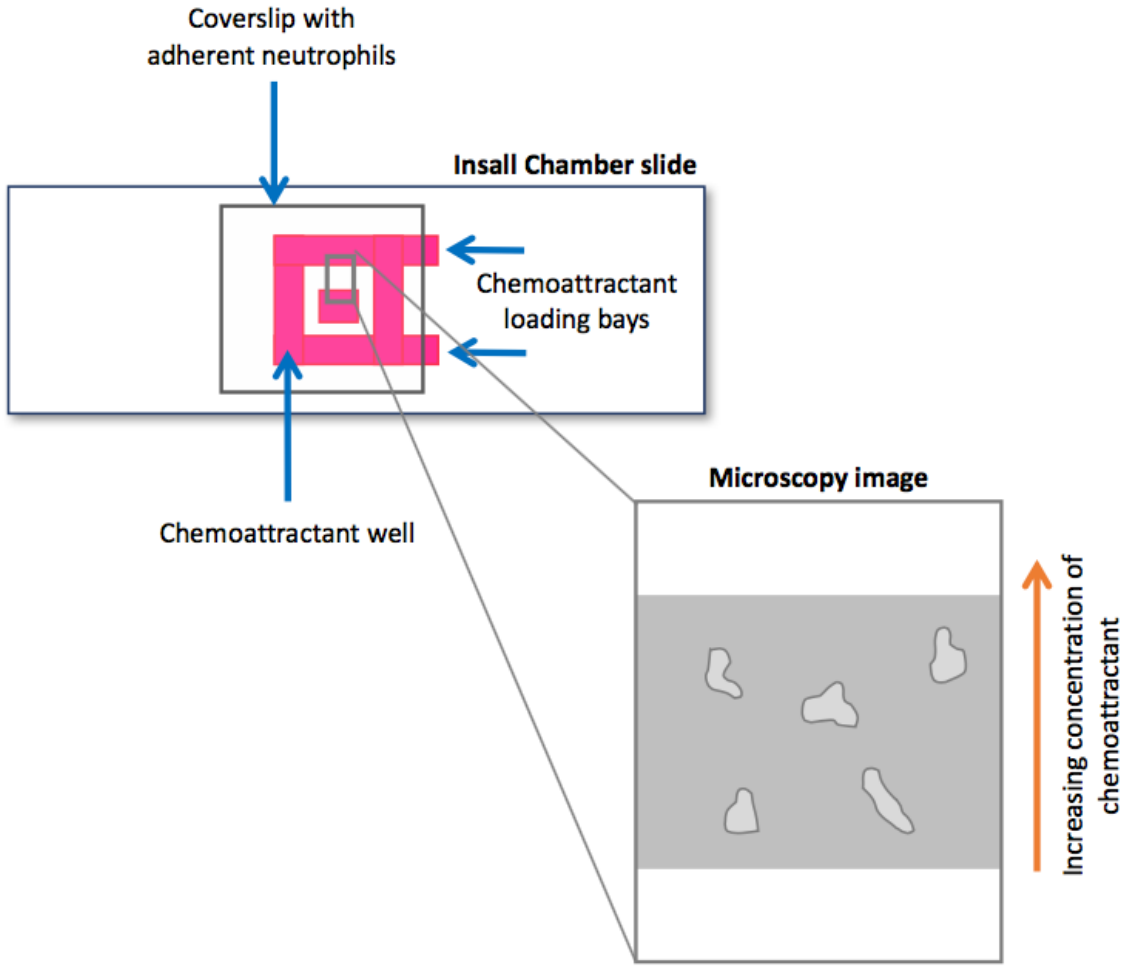
$2x10^5$ neutrophils suspended in RPMI-PS were seeded onto 22mmx22mm square glass cover slips (VWR International, UK) and incubated for a minimum of 30 minutes at 37°C in a humidified 5% CO_2 atmosphere to allow cells to adhere. Neutrophils were then stimulated with either RPMI-PS (negative control) of 25nM PMA (diluted in RPMI-PS) for 3 hours at 37°C, 5% CO_2. Cells were fixed by addition of 4% paraformaldehyde and a further 30 minute incubation at 37°C, 5% CO_2, then washed 3 times for 5 minutes in PBS at RT. Cells were permeabilised by incubation with 0.1% Triton X-100 for 1 minute at RT and then washed once in PBS as described above. DNA was stained using 1μM SYTOX® Green (Life Technologies, UK) dye (5 minutes, RT) and cells were washed a final time in PBS as described above. Specimens were then mounted in fluoromount medium and visualised using a LEICA DMI 6000 inverted microscope at x20 magnification.

2.2.19 Assessment of neutrophil migration

This was assessed using an Insall chamber (Weber Scientific International Ltd., Teddington, UK) as described previously (75, 260). This polymethyl methacrylate chamber consists of a central closed square chamber that is separated from a square outer chamber by two bridges of different thicknesses on opposite sides. This produces stable linear chemoattractant gradients of different steepness in a consistent and defined direction – the y direction (Figure 2-4).

Figure 2-4 Diagram representing neutrophil migration assay.

Insall chamber was loaded with RPMI-PS and chemoattractant after neutrophils were adhered to coverslip. Top (thick) bridge of chamber was visualised using light microscopy and movement of neutrophils in the direction of increasing concentration of chemoattractant was recorded.



Square glass 22mmx22mm coverslips were first coated with 400µl culture-tested 7.5% BSA (Sigma Aldrich, UK) and then with 400µl of a neutrophil suspension at $2x10^6$/ml in RPMI-PS with 0.15% v/v 7.5% BSA. After a 20 minute incubation at room temperature, the coated coverslip was inverted onto an Insall chamber. The chamber bridges are designed to lie 20-30µm below the surface of the coverslip when it is in position, thus

allowing cells to move towards the test chemoattractant. The chamber was filled with RPMI-PS (negative control) or the test chemoattractant diluted in RPMI-PS: 10nM fMLP (Sigma Aldrich, UK) or 100nM interleukin 8 (IL-8/CXCL8, R&D Systems, Abbingdon, UK).

Time-lapse recordings of cells at the wide chamber bridge were made using a LEICA DMI 6000 inverted microscope at x40 magnification. Recordings lasted 12 minutes per experiment, with 36 images captured per film using LEICA software. Cell tracking analysis was performed on 10 randomly selected neutrophils in the field of view using ImageJ software and manual tracking plug-in (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Neutrophil migration was assessed using 3 parameters as described in Table 2-6.

Table 2-6 Description of neutrophil migration parameters calculated in this study.

Chemokinesis / Speed (μm/min)	Average speed of cell movement: distance travelled in any direction over time between frames
Chemotaxis / Velocity (μm/min)	Average velocity of cell movement: speed in the y direction only (towards chemoattractant)
Chemotactic index / Accuracy	Vector analysis of cell movement: cosine of angle between orientation of cell and chemoattractant gradient at each frame

CHAPTER 3

CHARACTERISATION OF THE STUDY COHORT: DEMOGRAPHICS AND CLINICAL RESULTS

3.1 Introduction

The SONIC study was designed in order to evaluate immune system function in patients with CKD compared to age matched controls. Before moving on to describe differences in the immune system and its function, it is important to first describe the patient population, particularly with relation to severity of CKD. This chapter gives an overview of the demographic and clinical data of SONIC study participants (including comorbidities and medications), describes changes in participants' health status over the 6 months of the study and defines clinical laboratory parameters of the cohort including CKD severity. As described in Chapter 1 (Introduction), several clinical features and changes in laboratory parameters are associated with CKD that may exert immunomodulatory effects in addition to renal impairment. Analyses in this chapter identify such differences between controls and patients with CKD that may confound analysis of CKD-associated immune effects.

A total of 102 individuals (40 controls and 62 patients with CKD) were recruited to the SONIC study over 3 years (Figure 3-1), meeting the overall study sample size target. However, 36 participants (35%) were withdrawn from the study prior to vaccination with over a third due to patient choice (n=14, 39%). An unprecedented national shortage of PPV23 vaccines in the 2017-2018 vaccination season resulted in the withdrawal of 2 participants for whom the vaccines were not available on the day of the baseline visit. Despite thorough screening of hospital records prior to recruitment, exclusion criteria were subsequently met for 12 individuals (33%) from GP records, repeat blood samples and/or patient interview by the research team. Eight individuals (22%) had already received the seasonal trivalent influenza vaccine (TIV) in the community prior to attending the baseline SONIC visit (6 of these were in the first study vaccination season) and were therefore withdrawn from the study. This was despite both verbal and written advice (PIS) by the

research team that TIV would be given as part of the study. A substantial amendment of the PIS that emphasised this message to participants, together with a GP letter of study participation to explain that TIV would be given as part of the study, was subsequently approved and used for the following SONIC study seasons (2016 onwards).

A total of 66 individuals donated blood and urine samples at the baseline study visit (30 controls, 36 patients with CKD), which were used for cross-sectional analyses. One control participant subsequently declined vaccination and was therefore withdrawn from further follow-up. One patient with CKD was lost to follow-up after the baseline visit. Samples from only 29 controls and 35 patients with CKD were, therefore, available for longitudinal analyses following vaccination up to and including day 28. A further 5 patients with CKD and 1 control participant withdrew from the study prior to the month 6 follow-up visit.

3.2 Demographics and clinical parameters

Healthy controls and patients with CKD were matched for age and gender, but were mainly of White ethnicity (Table 3-1).

Figure 3-1 SONIC study consort diagram - flow chart of participants and withdrawals.

Blue colour denotes study flow, orange boxes denote withdrawn participants.

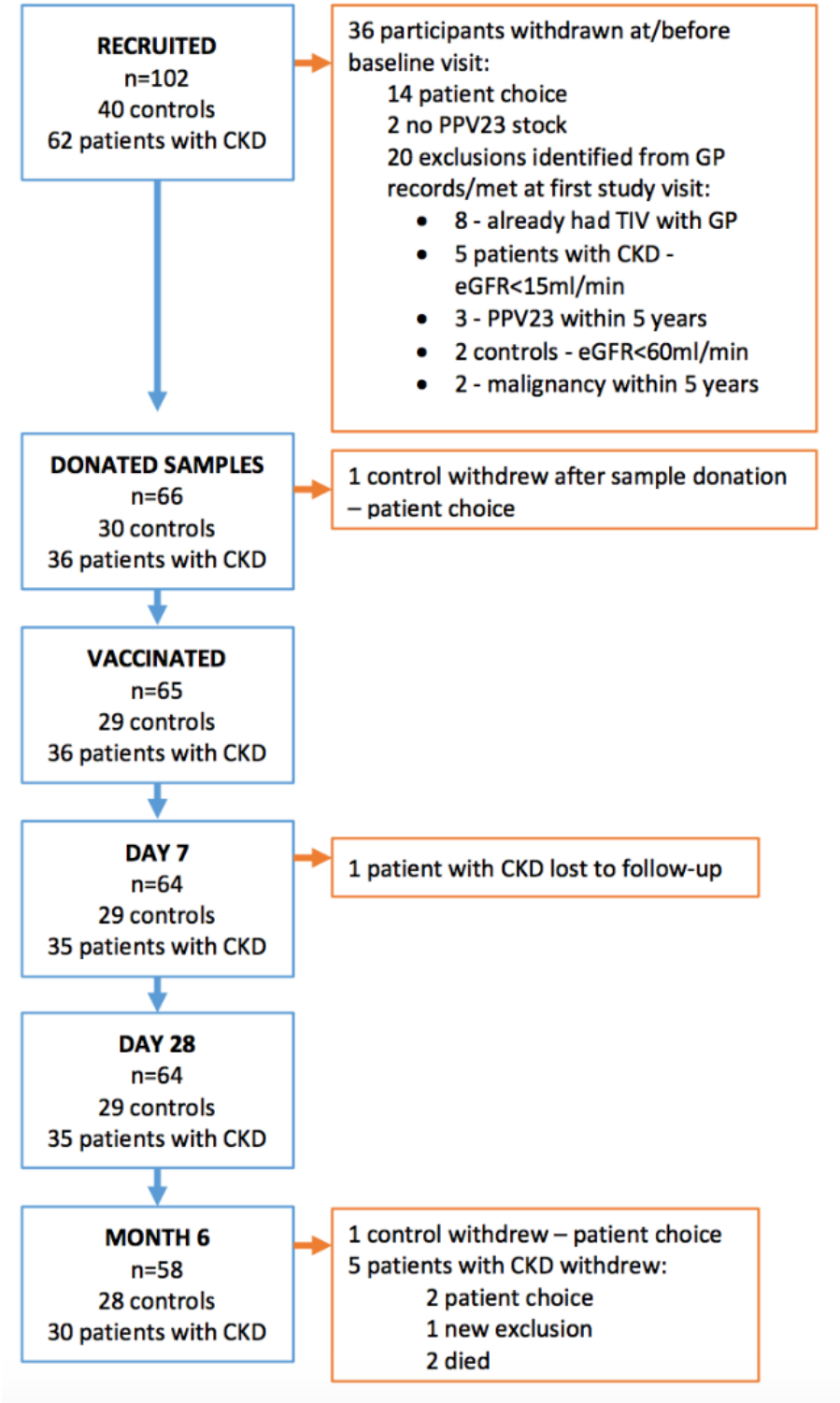


Table 3-1 Demographics of SONIC study participants.

Median and interquartile range shown unless stated. P value <0.05 considered significant. [a]Mann Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls n=30	CKD n=36	p value
Age, years	74 (11)	75 (12)	0.49[a]
Male: n (%)	15 (50)	16 (44)	0.80[b]
White ethnicity: n (%)	30 (100)	33 (92)	0.24[b]

3.2.1 Aetiology of renal disease

The most common identified cause of renal disease in patients with CKD in this study was ischaemic/hypertensive nephropathy (n=15, 42%). Five individuals had a diagnosis of diabetic nephropathy (14%), two individuals had obstructive uropathy (one of whom also had Fabry's disease), one had a congenital single kidney and one lithium-induced nephropathy. None of the patients with CKD in this study had immune-mediated renal disease, as per study exclusion criteria. However, a third of patients with CKD (n=12, 33%) in this study did not have a clearly defined aetiology for their renal disease. This was in the context of multiple comorbidities that are associated with renal impairment e.g. hypertension, atherosclerosis and diabetes. Patients with CKD had been under specialist nephrology follow-up for a median of 41 months (3.4 years) with a range of 3 months to 23 years.

3.2.2 Medical comorbidity and medications

As expected, patients with CKD were significantly more comorbid than controls (Table 3-2), with a significant excess prevalence of diabetes mellitus (DM), hypertension (HTN),

cardiac disease (ischaemic heart disease (IHD), congestive cardiac failure (CCF) and arrhythmias) and gastrointestinal conditions (mostly peptic ulceration and/or gastro-oesophageal reflux).

Table 3-2 Comorbidities of SONIC study participants.

N and (%) shown unless stated. DM: diabetes mellitus; HTN: hypertension; IHD: ischaemic heart disease; CCF: congestive cardiac failure; AF: atrial fibrillation; PPM: permanent pacemaker; CVA: cerebrovascular accident; TIA: transient ischaemic attack; COPD: chronic obstructive pulmonary disease; OA: osteoarthritis; BPH: benign prostatic hypertrophy. P value <0.05 considered significant. [a]Mann Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls n=30	CKD n=36	p value
Charlson Comorbidity Index: median (IQR)	**1 (1.25)**	**4.5 (3)**	**<0.0001[a]**
DM	1 (3)	24 (67)	**<0.0001[b]**
HTN	10 (33)	35 (97)	**<0.0001[b]**
IHD	0 (0)	16 (44)	**<0.0001[b]**
CCF	0 (0)	10 (28)	**0.001[b]**
Arrhythmia, including AF and PPM	3 (10)	14 (39)	**0.01[b]**
Cerebrovascular disease (CVA/TIA)	3 (10)	4 (11)	1.00[b]
Peripheral vascular disease	1 (3)	6 (17)	0.12[b]
Chronic respiratory disease, including COPD/asthma	1 (3)	7 (19)	0.06[b]
Previous malignancy, excluding non-melanoma skin cancer	3 (10)	5 (14)	0.72[b]
Hypercholesterolaemia	11 (37)	21 (58)	0.09[b]
Gastrointestinal, including peptic ulceration	3 (10)	18 (50)	**0.0005[b]**
Musculoskeletal, including gout/OA	12 (40)	23 (64)	0.08[b]
Metabolic, including hypothyroidism	4 (13)	4 (11)	1.00[b]
Urological, including incontinence/BPH	5 (17)	8 (22)	0.76[b]
Eye, including glaucoma/cataracts	3 (10)	7 (19)	0.33[b]
Mental health, including depression/anxiety	3 (10)	3 (8)	1.00[b]

A total of 8 participants had a history of malignancy >5 years prior to study enrolment without evidence of recurrence/metastasis (2: prostate cancer, 2: breast cancer, 2: colorectal cancer; 2: melanoma).

As expected with greater comorbidity, patients with CKD were prescribed significantly more medications per person than controls and the proportion of individuals with polypharmacy (an independent predictor of mortality/frailty (261, 262)) was significantly greater (Table 3-3). In parallel to a greater prevalence of cardiovascular disease and arrhythmias, significantly more patients with CKD were prescribed anti-platelet agents, anticoagulants and anti-anginal medications than controls. A slightly greater proportion of individuals with CKD were prescribed statin therapy than controls (known to modulate the immune system (263, 264)), but this did not reach statistical significance. Hypertensive individuals with CKD were prescribed a significantly greater number of anti-hypertensive agents per person than hypertensive controls, with a significant excess of second-line medications: diuretics, beta-blockers and doxazosin. This is not unexpected, as we know that renal impairment causes hypertension, which further worsens renal impairment in a positive feedback mechanism. Blood pressure control becomes more difficult with advancing renal impairment, frequently requiring a multiple anti-hypertensive medications and the use of less frequently used second-line agents.

Table 3-3 Medications prescribed for SONIC participants.

Median and IQR shown unless stated. NOAC: new oral anticoagulant; HTN: hypertension; DM: diabetes mellitus; PPI: proton pump inhibitor; H2RA: histamine 2 receptor antagonist. P value <0.05 considered significant. [a]Mann-Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls (n=30)	CKD (n=36)	p value
Number of medications per person	2 (2.25)	9 (4)	**<0.0001**[a]
Participants with ≥5 medications (polypharmacy): n (%)	4 (13)	35 (97)	**<0.0001**[b]
Anti-platelet agent (aspirin/clopidogrel): n (%)	3 (10)	14 (39)	**0.01**[b]
Anticoagulant (warfarin/NOAC): n (%)	1 (3)	11 (31)	**0.008**[b]
Statin: n (%)	12 (40)	23 (64)	0.08[b]
Other cholesterol-lowering therapy: n (%)	1 (3)	6 (17)	0.12[b]
Anti-anginals: e.g. nitrates, nicorandil, ivabradine: n (%)	0 (0)	10 (28)	**0.001**[b]
Inhalers/carbocysteine: n (%)	1 (3)	6 (17)	0.12[b]
Number of antihypertensives per person (of those with HTN)	1 (0.5)	3 (1)	**0.0002**[a]
Diuretic: n (% of HTN)	1 (10)	24 (69)	**0.002**[b]
Ca2+ channel blocker: n (% of HTN)	4 (40)	15 (43)	1.0[b]
Beta-blocker: n (% of HTN)	1 (10)	18 (51)	**0.03**[b]
ACEi/ARB: n (% of HTN)	5 (50)	13 (37)	0.49[b]
Doxazosin: n (% of HTN)	0 (0)	15 (43)	**0.02**[b]
Diabetes: oral anti-hyperglycaemics: n (% of DM)	1 (100)	8 (33)	0.36[b]
Diabetes: insulin: n (% of DM)	0 (0)	13 (54)	0.48[b]
Anti-acid therapy - PPI/H2RA: n (%)	1 (3)	17 (47)	**<0.0001**[b]
Regular analgesics: n (%)	3 (10)	19 (53)	**0.0002**[b]
Opiates: n (% of regular analgesics)	1 (33)	6 (32)	1.0[b]
Anti-gout therapy: n (%)	0 (0)	11 (31)	**0.0006**[b]
Oral iron: n (%)	0 (0)	6 (17)	**0.03**[b]
Folic acid: n (%)	0 (0)	3 (8)	0.24[b]
Vitamin B12: n (%)	1 (3)	1 (3)	1.0[b]

A third of diabetic patients with CKD were taking oral anti-hyperglycaemics and approximately half were insulin-dependent, suggesting difficult glycaemic control and/or prolonged duration of disease. Almost half of all patients with CKD were prescribed a

gastric acid lowering treatment e.g. proton pump inhibitor or ranitidine, compared with only one control, mirroring the observed markedly greater prevalence of gastrointestinal comorbidity in patients with CKD. Significantly more patients with CKD were taking regular analgesics (paracetamol +/- opiates) than controls, mirroring the slightly increased prevalence of musculoskeletal comorbidities compared to controls. Moderate-severe renal impairment is associated with hyperuricaemia and just under a third of patients with CKD were prescribed anti-gout therapy at the start of the study.

As discussed in Chapter 1 (Introduction) – CKD is also associated with anaemia, bone mineral disease and metabolic acidosis. Patients with CKD had significantly greater prescribing of oral iron than controls, but only one individual was prescribed ESA therapy at the start of the study. Just over a third of patients with CKD were prescribed 1-αcalcidol (n=13, 36%) and 11% (n=4) were prescribed sodium bicarbonate.

As per study exclusion criteria, none of the study participants had any active immune-mediated disease or were taking immunosuppressive medications.

3.2.3 BMI, smoking status and alcohol intake

BMI was available for 33 patients with CKD (92%), but only 14 controls (47%). In this dataset, BMI was significantly elevated in patients with CKD, with almost two thirds of the CKD group classified as obese or severely obese (BMI≥30 kg/m^2) – Table 3-4.

Table 3-4 Body mass index (BMI) of SONIC study participants.

Median and IQR shown unless stated. P value <0.05 considered significant and highlighted in bold. [a]Mann Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls n=14	CKD n=33	p value
BMI (kg/m^2)	24.7 (5.6)	31.0 (9.2)	**0.01**[a]
Underweight (BMI<18.5): n (%)	1 (7)	3 (9)	1.0[b]
Normal weight (18.5≤BMI<25): n (%)	7 (50)	3 (9)	**0.003**[b]
Overweight (25≤BMI<30): n (%)	4 (29)	6 (18)	0.46[b]
Obese (30≤BMI<40): n (%)	2 (14)	17 (52)	**0.02**[b]
Severely obese (BMI≥40): n (%)	0 (0)	4 (12)	0.30[b]

BMI significantly correlated with both CCI (Spearman's correlation coefficient 0.52, p=0.0002) and medication burden (Spearman's coefficient 0.35, p=0.02) in this study.

Significantly more patients with CKD identified themselves as "ever-smokers" than controls (Table 3-5), but smoking exposure (measured in pack years) was equivalent between the groups. "Ever-smokers" had a significantly higher CCI than individuals that had never smoked (medians 3.5 and 1, respectively; Mann Whitney 2-tailed p=0.0002).

Table 3-5 Smoking history of SONIC study participants.

Median and IQR shown unless stated. P value <0.05 considered significant. [a]Mann Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls n=30	CKD n=36	p value
Never smoked: n (%)	19 (63)	8 (22)	**0.001**[b]
Current smoker: n (%)	0 (0)	1 (3)	1.00[b]
Current vaper: n (%)	0 (0)	1 (3)	1.00[b]
Ex-smoker: n (%)	11 (37)	26 (72)	**0.006**[b]
Pack years (ever smokers)	10 (17.5)	15 (35)	0.68[a]

Smoking status and obesity (together with its' associated metabolic syndrome) are known to be associated with greater cardiovascular disease risk (265-267), so it is not surprising that these features were more prevalent in patients with CKD.

Control subjects had a significantly higher proportion of alcohol drinkers than patients with CKD (Table 3-6), but the degree of alcohol intake was equivalent between the groups. Interestingly, approximately 1 in 5 controls and patients with CKD identified as heavy drinkers (intake of over 14 units of alcohol per week), with a median of 20.5 units/week in both groups.

Table 3-6 Alcohol intake of SONIC study participants.

Median and IQR shown unless stated. P value <0.05 considered significant and highlighted in bold. [a]Mann Whitney 2-tailed p value; [b]Fisher's exact 2-tailed p value.

	Controls n=30	CKD n=36	p value
Alcohol: current drinkers: n (%)	26 (87)	21 (58)	**0.02**[b]
Alcohol intake of current drinkers: units/week	7.5 (2.5)	6 (18)	0.76[a]
Heavy drinkers (>14 units/week): n (%)	6 (20)	8 (22)	1.00[b]
Alcohol intake of heavy drinkers: units/week	20.5 (12.3)	20.5 (5.5)	0.51[a]

"Current drinkers" had generally higher lower CCI than "tee-total" individuals, which approached significance (Mann Whitney 2-tailed p=0.07). This is in keeping with previous observations that older adults that report greater health-related quality of life and lower incident frailty consume more alcohol than their more comorbid counterparts (268, 269).

3.2.4 Self-reported health events

Two-thirds of SONIC participants reported health events during 6 months of follow-up (n=43). These were defined as a new health condition (including infections) or diagnosis not previously known, an exacerbation of a pre-existing condition and/or any hospitalisation or medical procedure. A slightly greater proportion of patients with CKD reported health events than controls, but this did not reach statistical significance. Although the number of events reported by patients with CKD was almost twice that reported by controls (Table 3-7), time-adjusted incidence was not significantly different between the patient groups (rate ratio 1.5, 95% CI 0.91-2.59, mid P exact p=0.11).

Table 3-7 Self-reported new health events by SONIC study participants.

ENT: ear, nose and throat. P value <0.05 considered significant. [a]Fisher's exact 2-tailed value, .[b]mid P exact 2-tailed p value.

	Controls n=29	**CKD n=35**	**p value**
Participants reporting any health events: n (%)	18 (62)	26 (74)	0.42[a]
Participants reporting hospital admission: n (%)	3 (10)	6 (17)	0.49[a]
Number of health events reported over 6 month follow-up	**22**	**41**	
Time-adjusted reported incidence of health events (per 1000 person days, 95% CI shown)	**4.0 (2.5-6.1)**	**6.1 (4.4-8.3)**	**0.11[b]**
Cardiovascular: n (% of total)	2 (9)	2 (5)	0.61[a]
Respiratory: n (% of total)	7 (32)	20 (48)	0.29[a]
Gastrointestinal/urological: n (% of total)	6 (27)	5 (11)	0.17[a]
Neurological/ENT: n (% of total)	2 (9)	1 (2)	0.28[a]
Skin/musculoskeletal/dental: n (% of total)	2 (9)	11 (26)	0.15[a]
Trauma: n (% of total)	2 (9)	1 (2)	0.28[a]
Haematological: n (% of total)	1 (5)	0 (0)	0.35[a]
Malignancy: n (% of total)	0 (0)	2 (5)	0.54[a]

Overall, the majority of reported health events were respiratory (27 of 63 events, 43%) and infective in nature. Respiratory events made up a slightly greater proportion of health events reported in patients with CKD than controls. Patients with CKD reported a total of 11 upper respiratory tract infections (URTIs, 55% respiratory events), 8 lower respiratory infections (LRTIs)/infective exacerbations of COPD or asthma (40% respiratory events) and 1 new diagnosis of metastatic lung carcinoma. Control participants reported 5 URTIs (71% respiratory events), 1 LRTI and 1 new diagnosis of pulmonary fibrosis. A quarter of health events reported by patients with CKD were skin/musculoskeletal/dental – slightly greater than in controls – with 5 episodes of acute gout and one each of exacerbation of chronic lower back pain, dental abscess, cryotherapy to skin lesion, infected leg ulcers, broken down abdominal hernia skin and severe sunburn requiring dressings. Of the 2 events in this category reported by controls, one was a dental abscess and the other a new diagnosis of generalized eczema, treated with topical steroids.

Control participants reported a slightly greater proportion of gastrointestinal/urological events than patients with CKD, with one episode each of gallstone pancreatitis requiring inpatient admission, elective inguinal hernia repair, altered bowel habit with subsequent negative endoscopy, urinary tract infection (UTI) treated with oral antibiotics, severe gastro-oesophageal reflux and traveller's diarrhoea. One patient with CKD underwent an elective trans-urethral resection of the prostate (TURP) and one had an emergency hospital admission with priapism.

Two individuals in both control and CKD groups reported cardiovascular health events, which were an ischaemic CVA (1 control, 1 CKD), a new diagnosis of hypertension (1 control) and a new diagnosis of angina (1 CKD). Of the neurological/ENT events, 1 control individual each reported new short term memory loss and a recurrence of benign

paroxysmal positional vertigo (BPPV) symptoms, and 1 patient with CKD reported recurrent epistaxis. Of the trauma events, 1 control sustained a deep cat bite requiring antibiotics and dressings, another control sustained a finger laceration necessitating A&E attendance and 1 patient with CKD was involved in a minor road traffic collision. One control participant was found to have an incidental iron deficiency anaemia on baseline study bloods, which was subsequently investigated further by their GP. Two patients with CKD had a new diagnosis of metastatic carcinoma (one of lung origin, one of unknown origin) during their 6-month follow-up and sadly passed away before the end of 3 years of study.

The control individual that developed pulmonary fibrosis and the two patients with CKD who were diagnosed with metastatic carcinoma during the course of the study (thus fulfilling exclusion criteria) were subsequently excluded from analysis of immune function.

3.2.5 Infective episodes

Twenty-four participants reported a total of 33 infective episodes during the course of the study (Table 3-8). Patients with CKD reported over twice as many episodes as controls, with a slight excess of respiratory tract infections. The number and proportion of infective episodes that were treated with antibiotics and/or required hospitalisation were low and not significantly different between the two study groups.

Time-adjusted reported incidence of infection in patients with CKD was almost twice that of controls and approached significance (rate ratio 1.9, 95% CI 0.9-4.1, mid P exact 2-tailed p=0.09). However, when respiratory infections were considered separately, patients

with CKD had a significantly greater time-adjusted incidence than controls (rate ratio 2.4, 95% CI 1.0-6.7, mid P exact 2-tailed p=0.05). Interestingly, individuals that reported any infections were significantly younger than those that did not (median 72 years (IQR 6) versus 78 (IQR 11), Mann-Whitney 2-tailed p=0.006) and this difference persisted when the study population was split by disease group. There were no significant differences in gender, CCI or laboratory parameters (Hb, WCC, eGFR, ACR, HbA1c, hsCRP or CMV IgG/CMV serostatus - described in detail later in this chapter) between individuals that did or did not report infections over 6 months.

Table 3-8 Self-reported infective episodes by SONIC study participants.

P value <0.05 considered significant and highlighted in bold. [a]Fisher's exact 2-tailed p value, [b]midP exact 2-tailed p value.

	Controls n=29	CKD n=35	p value
Participants reporting any infective episodes: n (%)	9 (31)	15 (43)	0.44[a]
Participants reporting ≥2 infective episodes: n (%)	1 (3)	6 (17)	0.12[a]
Infective episodes reported over 6 month follow-up (n)	**10**	**23**	
Respiratory: n (% of total)	6 (60)	18 (78)	0.40[a]
GI/hepatobiliary: n (% of total)	2 (20)	1 (10)	0.21[a]
Other (dental, urinary, skin): n (% of total)	2 (20)	3 (14)	0.63[a]
Infective episodes requiring antibiotics: n (% of total)	5 (50)	11 (52)	1.0[a]
Hospitalisations for infection: n (% of total)	1 (10)	2 (10)	1.0[a]
Time-adjusted reported incidence of ALL infections (per 1000 person days, 95% CI shown)	1.8 (0.9-3.9)	3.4 (2.2-5.2)	0.09[b]
Time-adjusted reported incidence of respiratory infections (per 1000 person days, 95% CI shown)	1.1 (0.4-2.4)	2.7 (1.6-4.3)	**0.05**[b]

3.3 Medication changes

Over half of patients with CKD reported changes in medications over 6 months of follow-up compared with just under a quarter of controls, with almost twice as many medication change events (Table 3-9). The most common class of medications altered was anti-hypertensives, accounting for just over a third of medication change events in both controls and patients with CKD. Six patients with CKD commenced new treatments for anaemia during 6 months of follow-up (2 started IV iron, 2 oral iron, 1 vitamin B12 and 1 ESA), compared with none of the controls. This is not unexpected, given the association of renal impairment with anaemia.

Table 3-9 Medication changes reported by SONIC participants over 6 months.

N (%) shown. P value <0.05 considered significant and highlighted in bold. [a]Fisher's exact 2-tailed p value; [b]Mid-P exact 2-tailed p value.

	Controls (n=29)	CKD (n=35)	p value
Number of participants reporting medication changes	7 (24)	19 (54)	**0.02[a]**
Number of medication change events	13	22	0.36[b]
Anti-hypertensives	5 (38)	8 (36)	1.0[a]
Anaemia	0 (0)	6 (27)	0.06[a]
Diabetes	0 (0)	1 (5)	1.0[a]
Analgesia/anti-gout	0 (0)	2 (9)	0.52[a]
Anti-platelets/anticoagulation	2 (15)	0 (0)	0.13[a]
Anti-cholesterol therapy e.g. statin	2 (15)	0 (0)	0.13[a]
Other	4 (31)	5 (23)	0.70[a]

3.4 Significant adverse events (SAEs)

An SAE was defined as per the NHS Health Research Authority (HRA) guidance as an untoward occurrence that fulfilled one of more of the following criteria:

- resulted in death
- was life-threatening
- required hospitalisation / prologation of existing hospital stay
- results in persistent or significant disability / incapacity
- was otherwise considered medically significant by the research team.

10 SAEs were reported during the 3 years of the SONIC study, all of which were deemed unrelated to the study intervention. Eight events were reported for patients with CKD and 2 events for controls (see Table 3-10).

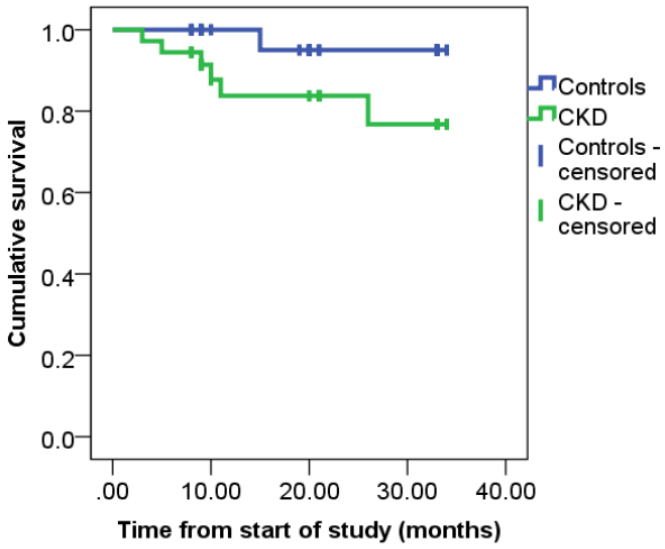
Two patients with CKD, but no controls died during the 6 months of study follow-up. A further 4 patients with CKD and 1 control participant who had died were identified when examining clinical notes during data collection/analysis at the end of the 3 years of study (August 2018). The difference in the mortality rate between controls and patients with CKD in this small study approached significance (Kaplan-Meier survival log-rank p=0.09, Figure 3-2).

Table 3-10 Summary of SONIC study SAEs by vaccination season.

IV – intravenous, ERCP – endoscopic retrograde cholangio-pancreatography, LRTI – lower respiratory tract infection, PAF – paroxysmal atrial fibrillation, AKI – acute kidney injury. Blue text refers to CKD patients, black text refers to controls.

	Type of SAE	Event description
2015-2016 vaccination cohort	Inpatient stay >1 night	Priapism requiring surgical intervention
	Inpatient stay >1 night	Gallstone pancreatitis requiring IV antibiotics and ERCP
	Inpatient stay > 1 night	LRTI requiring observation and oral antibiotics
	Prolonged hospital admission	Elective TURP – delayed discharge due to persistent haematuria and renal calculi
2016-2017 vaccination cohort	Inpatient stay >1 night	Cholecystitis requiring IV antibiotics
	Inpatient stay >1 night	New PAF and embolic stroke
	Inpatient stay >1 night	Sepsis and AKI, requiring IV antibiotics and dialysis
	Inpatient stay >1 night	Stroke and new diagnosis of metastatic lung cancer
	Unexpected death	Out of hospital cardiac arrest – likely secondary to myocardial infarction
2017-2018 vaccination cohort	Unexpected death	New diagnosis of metastatic cancer of unknown origin

Figure 3-2 SONIC study participant survival: Kaplan-Meier curve.



Of the patients with CKD who died, 3 suffered a cardiac arrest likely precipitated by an acute myocardial event and 2 died from metastatic carcinoma. One patient with CKD died from calciphylaxis, a life-threatening and rare syndrome typically associated with ESRD, characterised by intensely painful necrotic skin lesions that evolve as a result of microvascular ischaemia due to widespread vascular calcification (270). The control participant died from complications of pulmonary fibrosis diagnosed during their active participation in the SONIC study.

3.5 Clinical laboratory parameters

3.5.1 Haematology profile

Table 3-11 summarises the baseline clinical haematology parameters for SONIC study participants. Patients with CKD were significantly more anaemic than controls and had a significantly greater total white cell count, with higher neutrophil, monocyte and eosinophil counts (Figure 3-3). Patients with CKD were also slightly lymphopaenic compared to controls, but this did not reach statistical significance. These features are expected, as anaemia is a known complication of CKD and renal impairment is associated with chronic inflammation, with expansion of white blood cells, particularly neutrophils (193). In line with previous literature (208-210), patients with CKD had a significantly higher neutrophil/lymphocyte ratio (NLR) than controls. Interestingly, there was no significant difference in NLR between patients with both DM and CKD and those with CKD only.

The haematology profile of individuals was stable over a follow-up of 6 months, with no significant differences seen between measurements at baseline and month 6 in either controls or patients with CKD.

Table 3-11 Summary of baseline haematology parameters of SONIC participants.

Median and IQR shown unless stated. Hb: haemoglobin; MCV: mean cell volume; WCC: white cell count. P value <0.05 considered significant and highlighted in bold. [a]Mann Whitney 2-tailed p value, [b]unpaired t-test 2-tailed p value.

	Controls (n=30)	CKD (n=36)	p value	Reference range (adult)
Hb, g/L	140 (11)	116 (19)	**<0.0001**[a]	135-180 (male) 115-165 (female)
MCV, fL	92.9 (7.6)	93.0 (7.8)	0.86[b]	80.0-99.0
WCC, 10^9/l	6.0 (2.9)	7.2 (3.2)	**0.0008**[b]	4.0-11.0
Neutrophils, 10^9/l	3.3 (1.7)	4.9 (2.5)	**<0.0001**[a]	2.0-7.5
Lymphocytes, 10^9/l	1.6 (1.0)	1.3 (1.0)	0.08[a]	1.0-4.0
Monocytes, 10^9/l	0.4 (0.3)	0.6 (0.4)	**0.02**[a]	0.2-0.8
Eosinophils, 10^9/l	0.1 (0.1)	0.2 (0.1)	**<0.0001**[a]	0-0.4
Basophils, 10^9/l	0 (0.1)	0 (0.1)	0.60[a]	0-0.2
Platelets, 10^9/l	218 (60)	192 (72)	0.08[a]	150-450
Neutrophil/lymphocyte ratio	2.1 (0.8)	3.8 (2.4)	**<0.0001**[a]	

3.5.2 Biochemistry profile

Table 3-12 summarises the baseline clinical biochemistry parameters of SONIC participants. By design, patients with CKD had significantly higher urea and creatinine, mirrored by lower eGFR, than controls (Figure 3-4 – panels A-D). A small number of individuals had a calculated eGFR just below 15 at baseline, which was reported as 15 by the clinical laboratory (rounded up to 2 significant figures). As expected, proteinuria was significantly greater in patients with CKD (Figure 3-4 panel E) and increasing ACR was significantly associated with reducing eGFR (Spearman p<0.05).

Figure 3-3 Summary of haematology profiles of SONIC study participants – comparison between controls and patients with CKD.

Hb shown for males (A) and females (B), together with total white cell count (WCC – panel C), neutrophil count (D), lymphocyte count (E), neutrophil/lymphocyte ratio (NLR – panel F), monocyte (G) and eosinophil counts (H). Dashed red lines denote limits of normal range. Error bars denote median and IQR. *denotes $p<0.05$.

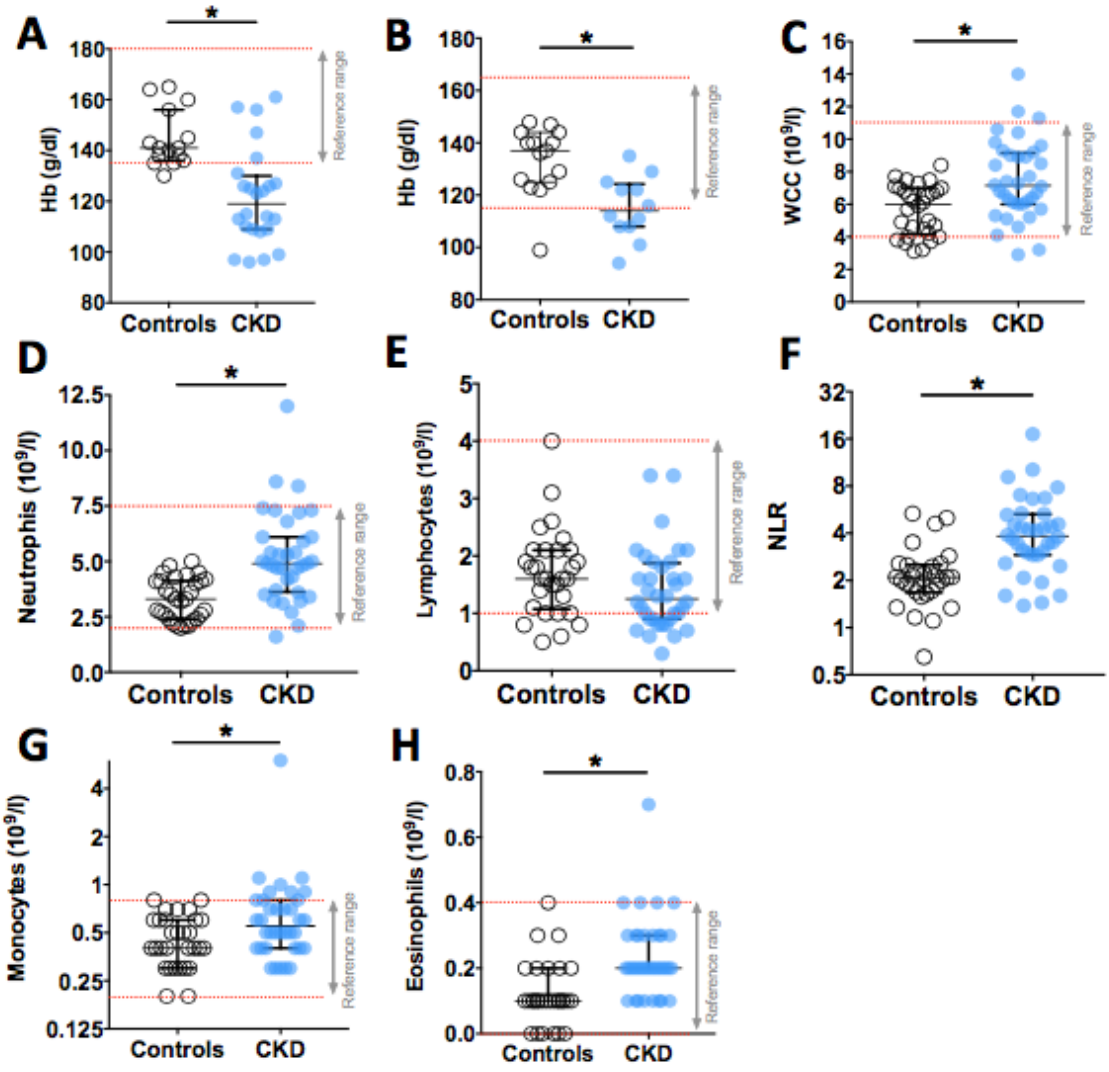


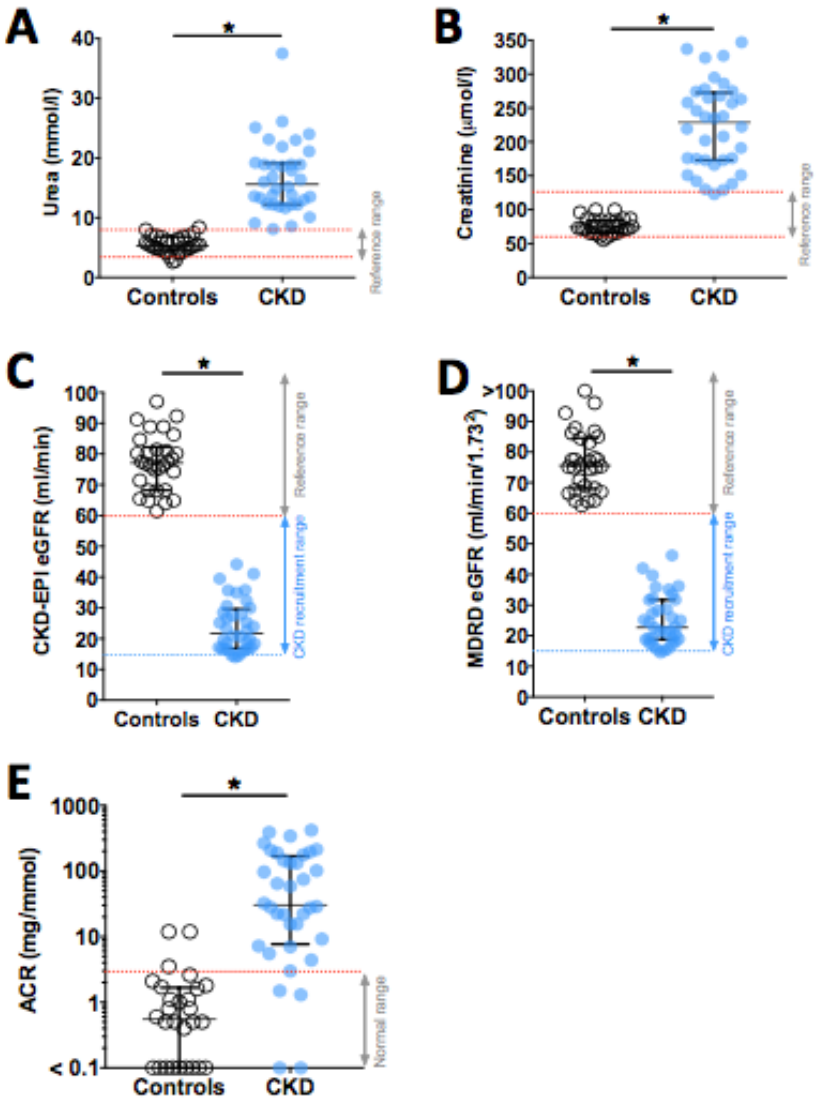
Table 3-12 Summary of baseline biochemistry parameters of SONIC participants.

Median and IQR shown unless stated. eGFR: estimated glomerular filtration rate; ACR: albumin/creatinine ratio; hsCRP: highly sensitive C-reactive protein; PTH: parathyroid hormone; TIBC: total iron binding capacity; HbA1c: glycated haemoglobin. P value <0.05 considered significant and highlighted in bold. [a]Mann Whitney 2-tailed p value, [b]unpaired t-test 2-tailed p value.

	Controls (n=30)	CKD (n=36)	p value	Reference range (adult)
Sodium, mmol/l	142 (3)	140 (5)	0.30[a]	134-146
Potassium, mmol/l	4.4 (0.4)	4.6 (0.9)	0.41[a]	3.4-5.2
Urea, mmol/l	5.4 (2.4)	15.6 (6.9)	**<0.0001**[a]	3.4-8.0
Creatinine, µmol/l	74 (19)	229 (100)	**<0.0001**[b]	60-126
eGFR (MDRD)	76 (16)	23 (13)	**<0.0001**[a]	>60
eGFR (CKD-EPI)	77 (14)	22 (13)	**<0.0001**[a]	>60
ACR, mg/mmol	0.6 (1.7)	30.5 (160.5)	**<0.0001**[a]	<3
hsCRP, mg/l	0.84 (1.17)	4.96 (4.70)	**<0.0001**[a]	<3
Albumin, g/l	47 (4)	45 (5)	**0.002**[b]	34-51
Total protein, g/l	72 (7)	72 (8)	0.85[b]	60-80
Alkaline phosphatase, U/l	71 (29)	80 (30)	0.22[a]	40-130
Phosphate, mmol/l	1.14 (0.22)	1.18 (0.25)	0.40[b]	0.80-1.40
Calcium, mmol/l	2.43 (0.15)	2.41 (0.16)	0.47[b]	2.10-2.60
PTH, pmol/l	-	12.7 (8.0)	-	1.6-6.9
Vitamin B12, ng/l	438 (280)	363 (180)	0.33[a]	200-900
Folate, µg/l	9.5 (7.6)	5.7 (5.0)	**0.001**[a]	2.7-18.8
Iron, µmol/l	18.3 (7.2)	11.6 (5.0)	**<0.0001**[b]	10.0-32.0
Ferritin, µg/l	114 (97)	125 (143)	0.21[a]	18-360
TIBC, µmol/l	61.2 (10.5)	57.9 (16.8)	0.56[b]	
Transferrin, g/l	2.68 (0.43)	2.48 (0.66)	0.19[b]	2.00-3.60
Iron saturation (%)	30.4 (11.4)	20.1 (6.9)	**0.0001**[b]	
HbA1c, mmol/mol	37 (4)	50 (22)	**<0.0001**[a]	<48 in non-diabetics

Figure 3-4 Summary of renal profile of SONIC study participants – comparison between controls and patients with CKD.

Urea (A), creatinine (B), eGFR (CKD-EPI in panel C, MDRD in panel D), albumin/creatinine ratio (ACR) – panel E. Dashed red lines denote limits of normal range. Error bars denote median and IQR. *denotes $p<0.05$.



Over a third of patients with CKD had severe, G4A3 stage, disease (summarised in Table 3-13 and Figure 3-5).

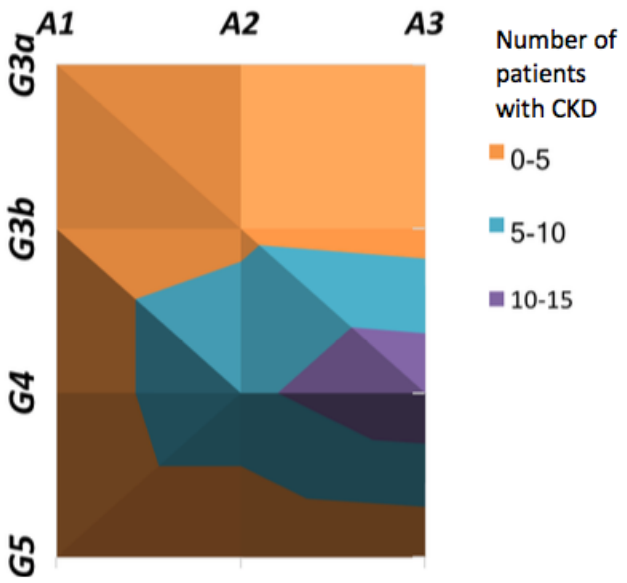
Table 3-13 Classification of CKD disease in patients with CKD by eGFR and ACR.

N and % shown. Grey text denotes classification based on CKD-EPI eGFR, black text denotes MDRD eGFR.

			Persistent albuminuria categories (mg/mmol)		
			A1 <3	A2 3-30	A3 >30
		TOTAL	4 (11)	14 (39)	18 (50)
GFR categories (ml/min/1.73²)					
G3a	45-59	1 (3) 1 (3)	0 (0) 1 (3)	1 (3) 0 (0)	0 (0) 0 (0)
G3b	30-44	9 (25) 7 (19)	2 (6) 1 (3)	4 (11) 3 (8)	3 (8) 3 (8)
G4	15-29	25 (69) 25 (69)	2 (6) 2 (6)	9 (25) 11 (31)	14 (39) 12 (33)
G5	<15	1 (3) 3 (8)	0 (0) 0 (0)	0 (0) 0 (0)	1 (3) 3 (8)

Figure 3-5 Density plot summarising classification of CKD disease in SONIC patients with CKD.

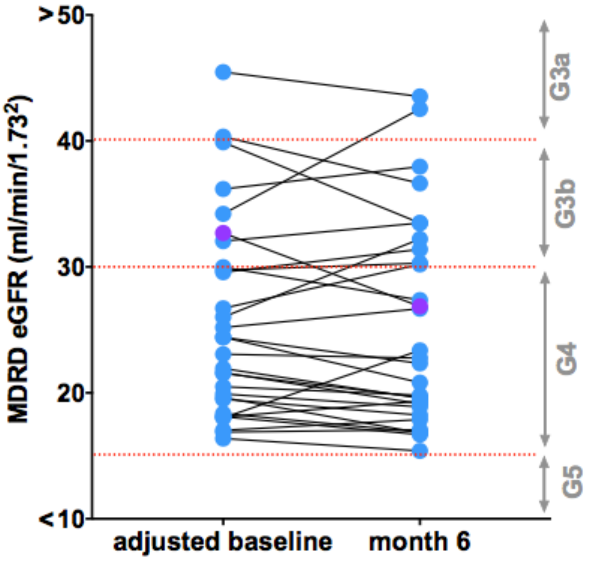
MDRD eGFR shown as representative.



There was no significant change in eGFR over 6 months in patients with CKD overall. Progression of CKD is defined as a sustained drop in eGFR of 25% or more and a change in GFR category over 1 year, and/or a sustained drop in eGFR of >15ml/min over 1 year (99). If these parameters are halved and applied to SONIC patients with CKD (assuming a constant rate in renal function decline), only one individual fulfils criteria for progression (Figure 3-6). This patient died of an acute myocardial infarction 18 months after completing SONIC study follow-up.

Figure 3-6 Change in eGFR (MDRD shown) over 6 months.

Purple colour denotes data from progressor patient. Adjusted baseline: mean taken of B and F1 results to account for natural variability in eGFR; dashed red lines shown limits of eGFR for CKD G stages.



In keeping with the greater prevalence of diabetes in patients with CKD, HbA1c was also significantly higher in this patient group (Figure 3-7 A). As expected, hsCRP - a marker of inflammation, was significantly greater in patients with CKD than controls (Figure 3-7 B), mirroring the pattern seen with neutrophils and NLR. Albumin was significantly lower in patients with CKD than controls (Figure 3-7 C), in keeping with a chronic inflammatory process. Although calcium and phosphate levels were similar between patients with CKD and controls, median PTH in patients with CKD was above the reference range for normocalcaemic individuals, in keeping with what is known about bone mineral dysregulation with CKD.

Interestingly, folate and iron levels (but not vitamin B12) were significantly lower in patients with CKD compared to controls, despite receiving supplementation (3 patients with CKD for folate, 6 for oral iron).

3.5.3 Relationships between haematology and biochemistry parameters

As expected, markers of renal disease severity were associated with markers of known complications of CKD. Significant negative correlations were seen between eGFR and markers of inflammation (WCC, neutrophil count, NLR and hsCRP) and bone mineral disease (alkaline phosphatase and phosphate). Significant positive correlations were seen between eGFR and markers of anaemia (Hb, folate, iron). The relationships seen with eGFR were mirrored by significant, but opposite relationships with ACR.

Figure 3-7 Measures of glycaemic control (A), hsCRP (B) and albumin (C) in patients with CKD compared to controls.

Dashed red lines denote limits of normal range. Error bars denote median and IQR. *denotes $p<0.05$.

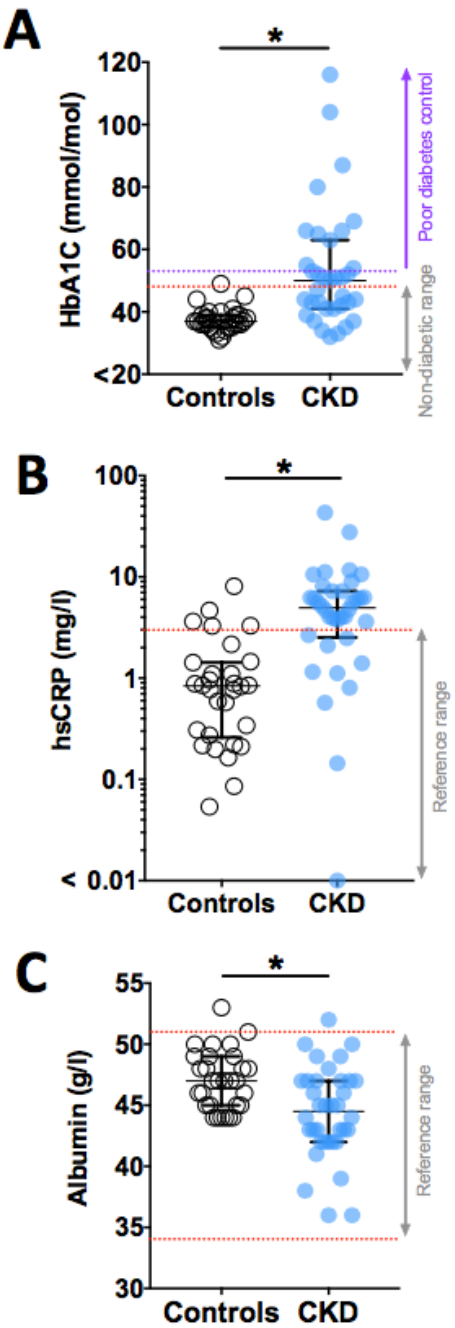
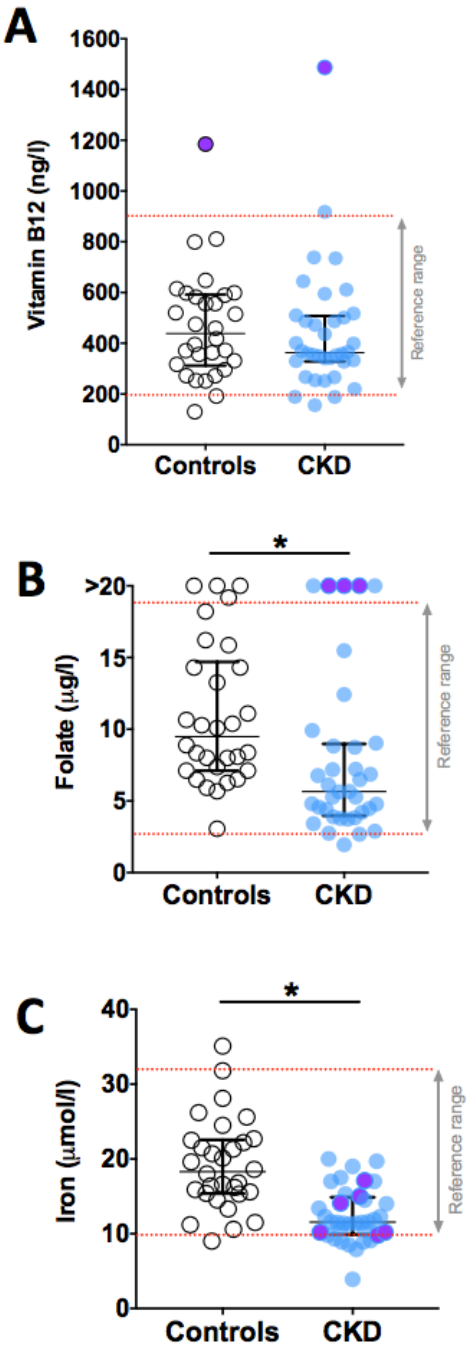


Figure 3-8 Summary of serum vitamin B12 (A), folate (B) and iron (C) in SONIC study participants – comparison between controls and patients with CKD.

Dashed red lines denote limits of normal range. Purple colour denotes data from individuals receiving supplementation. Error bars denote median and IQR. *denotes $p<0.05$.



3.6 Serum clinical immunology profile

3.6.1 Total immunoglobulins

Quantifying total circulating immunoglobulins (IgA, IgM and IgG) is an important means of assessing the immune system. Deficiency in any and all of these immunoglobulin classes are associated with a number of clinical syndromes of increased susceptibility to infection as described in Chapter 1 (Introduction).

Baseline serum samples from SONIC study participants recruited during the 2015/16 and 2016/17 vaccination seasons (20 controls, 25 patients with CKD) were tested for total immunoglobulins. No significant differences in total IgG, IgA and IgM serum concentrations were seen between controls and patients with CKD (Figure 3-9). Just over a third of controls and patients with CKD had low serum total concentrations of IgM (Table 3-14), which is expected as IgM is known to decline with age (271). Interestingly, 3 patients with CKD, but no controls, had low total serum IgG concentrations, and this class deficiency was not isolated - 2 individuals also had low total IgM, but normal IgA and 1 had low total IgA, but normal IgM.

In view of these finding, we then examined IgG subclass concentrations in a small subset of SONIC participants (11 controls, 14 patients with CKD – 2 of whom had low total IgG). There were no significant differences in serum concentrations of IgG1-4 between patients with CKD and controls (Figure 3-10).

Figure 3-9 Total serum immunoglobulins in SONIC study participants – comparison between controls and patients with CKD.

Serum concentrations of total IgA (A), IgM (B) and IgG (C). Dashed red lines denote limits of normal range; error bars denote median and IQR.

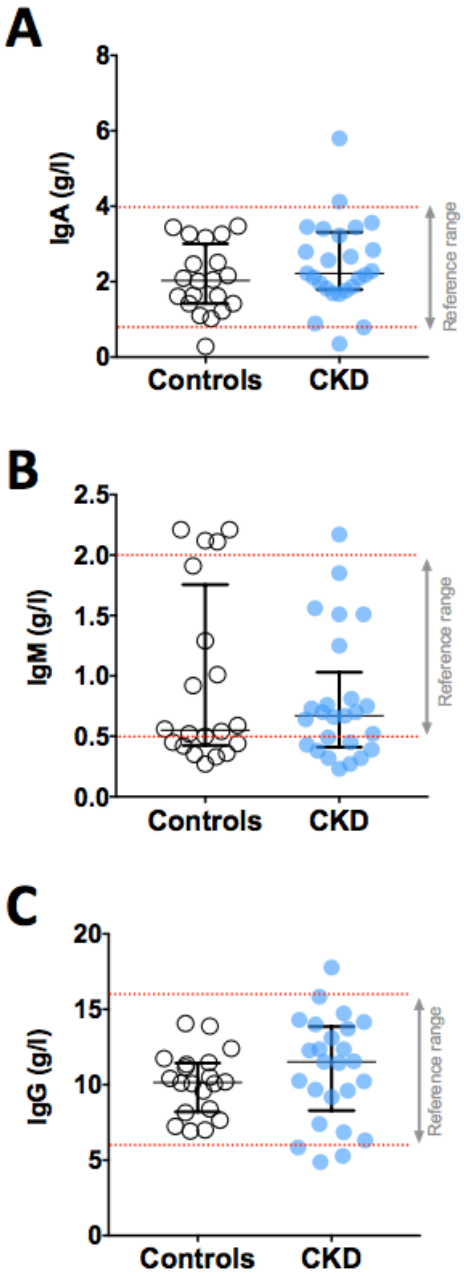


Table 3-14 Summary of SONIC participants with low total serum immunoglobulin levels.

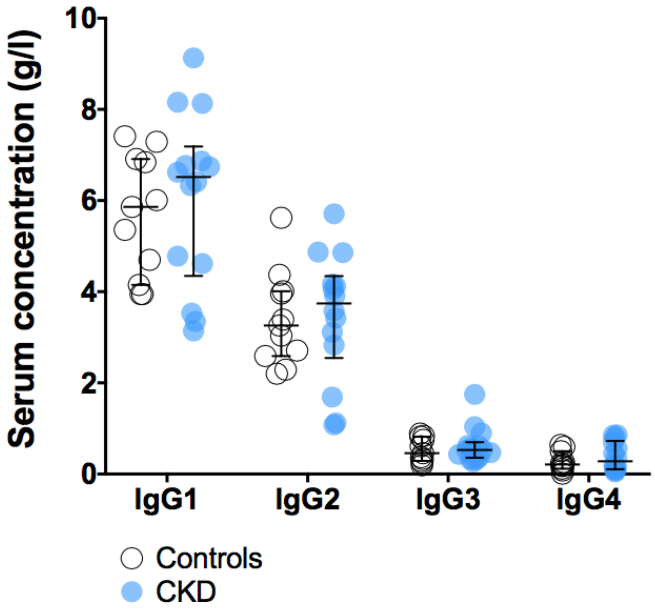
N (%) shown unless stated, Fisher's exact p shown. P value <0.05 considered significant.

	Controls (n=20)	CKD (n=25)	p value
Low IgG	0 (0)	3 (12)	0.24
Low IgA	1 (5)	2 (8)	1.0
Low IgM	7 (35)	9 (36)	1.0
1 or more low immunoglobulin classes	0 (0)	3 (12)	0.24

Figure 3-10 Serum concentrations of IgG subclasses in sample of SONIC study participants – comparison between patients with CKD and controls.

Blue symbols denote data from patients with CKD, unfilled symbols denote data from controls.

Error bars denote median and IQR.



3.6.2 Anti-TT/DT IgG

Our group has previously shown that antibody responses to several historic antigens in patients with CKD can be at least equivalent to healthy controls, which suggests that renal

impairment may not induce a global immunodeficiency state, but one that reflects the timing of antigen encounter (180). As such, I examined the impact of renal impairment on already established humoral immune memory in the SONIC cohort, by evaluating humoral responses to several antigens typically encountered by early adulthood: tetanus and diphtheria toxoids (TT and DT – vaccine antigens), and cytomegalovirus (CMV).

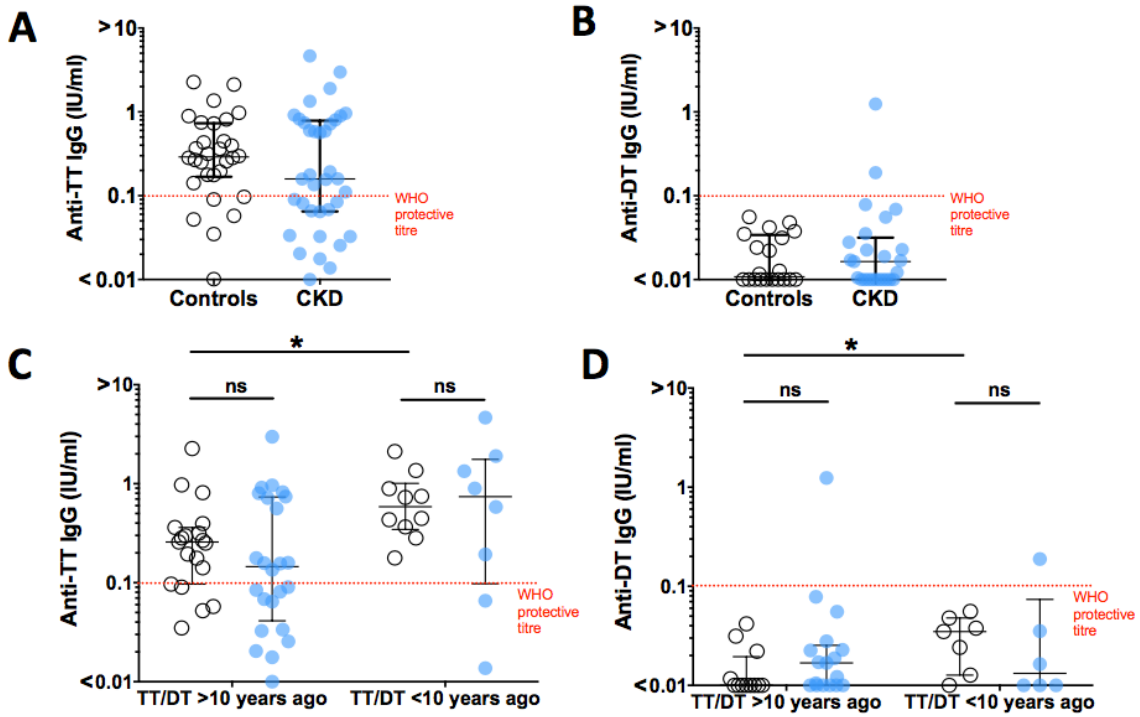
Tetanus/diphtheria (TT/DT) vaccination history was available for 29 controls and 31 patients with CKD (91% total study population). Eighteen SONIC study individuals (30%) had a TT/DT booster vaccination in the 10 years preceding their participation in the study and there was no significant difference in TT/DT coverage between patients with CKD (n=8, 26%) and controls (n=10, 34%). No significant differences were observed in anti-TT or anti-DT IgG titres between patients with CKD and controls (Figure 3-11) and this was unchanged if data from individuals with undetermined vaccination history were included. As expected, control participants that had received a TT/DT vaccine in the preceding 10 years had significantly higher anti-TT and anti-DT IgG titres than controls that had not had a booster vaccine. This pattern, however, was not seen in patients with CKD.

When clinical correlates of protection were considered, 22 patients with CKD (61%) had levels of anti-TT IgG above the WHO-defined threshold associated with tetanus protection (0.1μg/ml (272)) - hereafter referred to as “protective titre”, compared to 24 controls (80%, Fisher’s exact 2-tailed p=0.11). Only 2 patients with CKD (8%) and no controls (Fisher’s exact 2-tailed p=0.49) had levels of anti-DT IgG above the protective titre as defined by WHO (0.1μg/ml (273)).

There were no significant correlations between anti-TT IgG titres and measures of renal disease severity – eGFR and ACR - in all SONIC participants or when controls and patients with CKD were considered separately. Interestingly, anti-DT IgG significantly correlated with eGFR, but not ACR, in patients with CKD (Spearman's rank 2-tailed p=0.04, correlation coefficient 0.41), but no significant relationships were seen in controls.

Figure 3-11 Serum concentrations of anti-TT and DT IgG in SONIC study participants – comparison between controls and patients with CKD.

A - TT, B – DT: comparisons between controls and patients with CKD. C – TT and D – DT: comparisons between patient groups split by recent booster vaccination status. Error bars denote median and IQR, unfilled symbols denote data from controls, blue symbols denote data from patients with CKD. Dashed red line denotes WHO protective titre. *denotes p<0.05, ns – not significant.



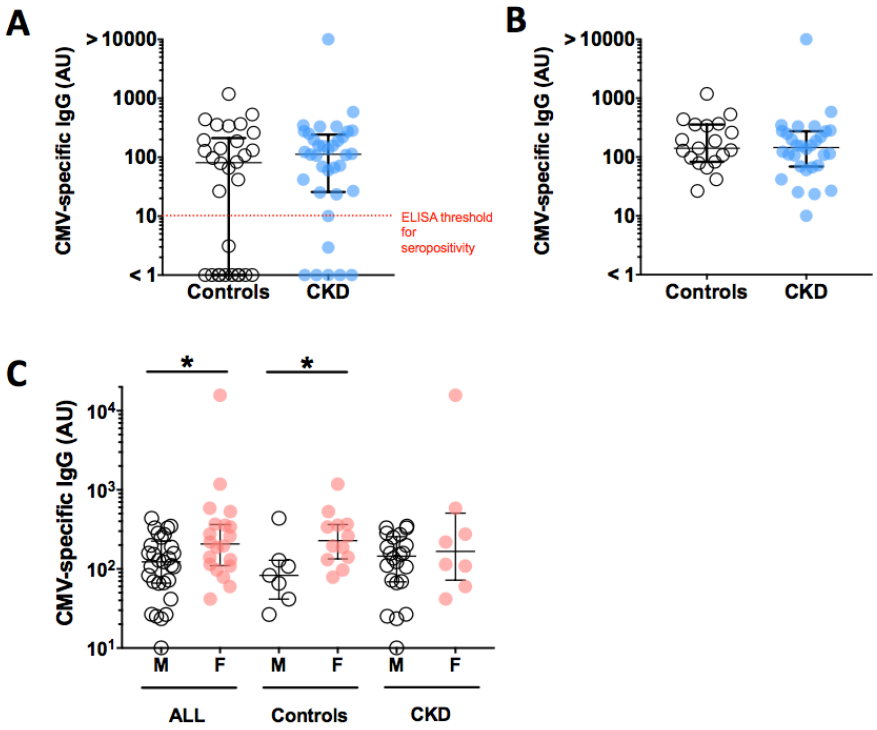
3.6.3 CMV-specific IgG

Serum levels of CMV-specific IgG were determined by ELISA at baseline for all SONIC participants and a titre of 10 AU or greater was used to determine CMV seropositivity.

Although patients with CKD had an excess of seropositive individuals compared to controls that approached significance (19 controls (63%) versus 30 patients with CKD (83%), Fisher's exact 2-tailed p=0.09), absolute titres of seropositive individuals were not significantly different between the two disease groups (Figure 3-12 A, B). However, a significantly greater proportion of male patients with CKD were seropositive for CMV than male controls (n=7 (47%) and n=22 (92%) respectively, Fisher's exact p=0.003) and seropositive females generally had higher titres than seropositive males (Figure 3-12 C). Interestingly, there were no significant differences in measures of multimorbidity (CCI, medication burden) when comparing male and female SONIC participants either as a whole study cohort or when split by disease group. However, male study participants had a significantly higher weekly alcohol intake than females overall (median 6 units (IQR 18.5) and median 3.5 (IQR 7), respectively, Mann Whitney 2-tailed p=0.009). Haematology and biochemistry laboratory parameters were also similar between SONIC study males and females, except for platelet and monocyte counts, where female participants had significantly higher platelet counts (median 240.5 (IQR 39) versus median 184.5 (IQR 54) for males, Mann Whitney 2-tailed p=0.002) and lower circulating monocytes (median 0.4 (IQR 0.2) versus median 0.6 (IQR 0.3) in males, Mann Whitney 2-tailed p=0.01) than males.

Figure 3-12 Serum CMV-specific IgG titre in SONIC study participants.

Comparison between controls and patients with CKD: A – all participants; B – CMV-seropositive only; C – CMV-seropositive individuals split by gender (rose colour denotes females). Error bars denote median and IQR. Dashed red line in panel A denotes threshold for CMV seropositivity. *denotes p<0.05.



3.7 Discussion

This Chapter characterises the demographic, clinical and basic laboratory parameters of the SONIC study population. This study has several strengths. Overall, the CKD and control groups were matched for age and gender. The CKD group was also broadly representative of the wider population of moderate/severe CKD patients, with a median eGFR of 23 ml/min and approximately a third of individuals classified as CKD G4A3

(moderate/severe renal impairment with severe proteinuria). The exclusion of dialysis therapy and immune-mediated/malignant health conditions in this study allows for a "cleaner" analysis of immune system phenotype and function in CKD than previous studies. However, study participants recruited were mainly of White ethnicity, and, as such, results may not be entirely applicable to the wider, more ethnically diverse CKD population.

Although the recruitment target of 100 study participants was reached by the end of the 3 years of the SONIC study, a large number of individuals were withdrawn prior to vaccination, resulting in a smaller sample size than planned – the main weakness of this study. However, this sample size approximates several other studies of immune function and phenotype in populations with renal disease (239, 274).

The majority of CKD patients had a diagnosis of ischaemic/hypertensive or diabetic nephropathy – the two most common non-immune causes of CKD in adults (100). In keeping with previous literature, patients with CKD in this study reported slightly more health events and significantly more respiratory infections than controls in a period of 6 months. Over the course of the 3 years of study, the CKD group also had a slightly higher mortality rate than controls.

As expected, patients with CKD were significantly more comorbid than controls with a greater prescribing of anti-hypertensive and other "cardiac" medications. However, there was a significantly greater prevalence of DM in the CKD group than expected (67% versus approximately 30% in the wider CKD population (114)). This is likely due to the study selection criteria of older adults, as DM prevalence (like CKD) increases with age (115). Interestingly, the control study participants were really very "well", with a median

CCI of 1 and hypertension, hypercholesterolaemia and musculoskeletal conditions e.g. OA/gout being the main comorbidities reported. As such, multimorbidity and, specifically, the presence of DM, are potential confounders in the subsequent analysis of CKD impact on immune system phenotype and function in this study.

Patients with CKD had clinical laboratory features in keeping with known complications of CKD – anaemia (with low serum iron and folate) and elevated inflammatory markers (neutrophilia, high NLR ratio and hsCRP). No significant differences were seen between controls and patients with CKD in total immunoglobulins and IgG titre to the historic vaccine antigens, TT and DT. A slightly greater proportion of patients with CKD had evidence of latent CMV infection compared to controls (83% versus 63%), but the CMV-specific IgG titre was equivalent between the groups in CMV seropositive individuals. As CMV infection is known to modulate the adaptive immune system (see Chapter 1, Introduction), this is another potential confounder in the analysis of CKD impact on the immune system in this study.

Multiple comparisons have been made between data from patients with CKD and controls in this chapter and although some of the significant findings may represent type I errors (false positive), most are probably due to genuine differences between the disease groups.

CHAPTER 4

VACCINE RESPONSES IN PATIENTS WITH CKD

4.1 Introduction

The presence of CKD and ESRD/dialysis therapy have previously been associated with impaired vaccine responses, particularly for hepatitis B, with studies consistently demonstrating lower peak antibody titre, lower seroconversion rates and faster decline in protective antibody titres than healthy individuals (157, 181, 253).

The effect of renal impairment on immune responses to various vaccines in older adults has not previously been systematically interrogated. The clinical study described in this thesis was designed to comprehensively evaluate the immune system in older patients with CKD, with a particular focus on adaptive immune function, where administration of clinically recommended seasonal vaccines (T-dependent seasonal trivalent influenza vaccine, TIV, and T-independent 23-valent pneumococcal polysaccharide vaccine, PPV23) acted as an *in vivo* antigen challenge. These vaccines were chosen specifically to examine both T-dependent and T-independent immune responses in the same individual.

Responses to seasonal influenza vaccines are generally lower in older adults than those seen in young adults (64, 275). Despite this, influenza vaccination in the elderly is generally associated with reduced winter morbidity/mortality on a population level (276). Seasonal influenza vaccine responses in adults with CKD (including those treated with dialysis) are variable (176, 277, 278) and vaccine efficacy in ESRD is unclear (279). However, given the significant negative effects of influenza disease in this vulnerable patient population, annual vaccination is recommended (42).

As with TIV, PPV23 responses in older and/or comorbid adults are generally lower and of poorer quality than in young adults (280) and vaccine effectiveness in these vulnerable populations is unclear (281). As described in Chapter 1 (Introduction),

hyporesponsiveness to repeat PPV23 vaccination (defined as the inability to mount an immune response upon re-exposure to antigen of at least the same or greater magnitude as that seen after primary exposure) is also a potential concern in this population as the inability to boost immune responses may limit both the effectiveness and the utility of the vaccine. Although PPV23 hyporesponsiveness has been observed in older adults (55, 56), its clinical impact remains under debate, particularly with evidence of vaccine efficacy in preventing the incidence of pneumococcal disease, even in adults aged over 50 years (282).

In this Chapter I will describe the humoral response elicited by TIV and PPV23 in patients with CKD compared to age and gender-matched controls. The conventional method of evaluating responses to vaccines is to measure circulating antigen-specific antibody before and after vaccination, with increases in concentrations attributed to vaccine-induced immune activation. Antigen-specific antibody titres are, by convention, reported as geometric means in population studies of vaccine response.

The gold-standard method of measuring humoral responses to TIV is by the haemagglutination inhibition assay (HAI, as described in Chapter 2, section 2.2.7) at baseline and day 28 post-vaccination for the specific influenza strains contained in the vaccine. This is a functional assay that evaluates the ability of antibody to haemagglutinin (HA – a surface protein of the influenza virus) to prevent erythrocyte agglutination by blocking HA binding to erythrocyte cell surface receptors. “HAI titre” is defined as the reciprocal of the highest serum dilution that completely inhibits haemagglutination in this assay. A variability in immunogenicity (the capacity to induce a robust immune response) can exist between different influenza strains from different vaccination seasons (283).

The gold-standard for PPV23 response assessment is the measurement of concentrations of anti-pneumococcal polysaccharide (PnPS) IgG at the same timepoints as for TIV (31). By convention, both HAI titres and anti-PnPS IgG concentrations are reported as geometric means when comparing responses between populations. Although PPV23 contains capsule polysaccharides from 23 different pneumococcal serotypes, there is little consensus on how many or which serotypes should be measured to examine vaccine response. The humoral response to PPV23 can be used in children and adults as a tool for diagnosis of antibody deficiencies (54) and guidelines for this recommend measurement of responses to a minimum of 4 serotypes, accepting that most centres examine between 12 and 14 (284). In this study we used an established clinical assay that was developed for assessment of immunodeficiency to examine responses to PPV23 (256) – see Chapter 2, section 2.2.6. This multiplexed assay measures IgG concentrations against 12 pneumococcal serotypes: 1, 3, 4, 5, 6b, 7f, 9V, 14, 18c, 19A, 19F and 23F.

The analysis presented here will address both the primary and secondary outcomes of the SONIC study: the proportion of CKD patients having adequate response and protective antibody titres following vaccination with TIV and PPV23 compared to healthy controls and the proportion of vaccinated individuals maintaining protective antibody titres 6 months following vaccination. For clarity, only data from baseline, day 28 and month 6 post-vaccination timepoints are presented. Day 7 antigen-specific HAI and IgG data was collected, but did not alter the patterns observed in humoral responses at day 28 and month 6 and is therefore not included in this Chapter.

4.2 Vaccination history

Coverage with the preceding season's TIV was high and similar between controls and patients with CKD across the 3 study seasons (Table 4-1).

Table 4-1 Preceding season's TIV coverage – controls v CKD.

Fisher's exact 2-tailed p values shown. Significance defined as $p<0.05$.

	Controls	CKD	p value
2015-2016 season	n=10	n=13	
Recipients of 2014-2015 TIV: n (%)	8 (80)	12 (92)	0.56
2016-2017 season	n=9	n=10	
Recipients of 2015-2016 TIV: n (%)	8 (89)	9 (82)	1.0
2017-2018 season	n=9	n=10	
Recipients of 2016-2017 TIV: n (%)	8 (89)	9 (90)	1.0
ALL seasons	n=28	n=33	
Received TIV in preceding season: n (%)	24 (86)	30 (91)	0.69

However, coverage with PPV23 was significantly lower in controls than patients with CKD (Table 4-2). Median time from previous PPV23 was approximately 10 years and not significantly different between the two study groups.

Table 4-2 PPV23 vaccination history of SONIC study participants.

Median and IQR shown unless stated. PPV23: 23-valent pneumococcal polysaccharide vaccine; [a]Fisher's exact 2-tailed p value, [b]Mann Whitney 2-tailed p value; significance defined as $p<0.05$.

	Controls n=28	CKD n=33	p value
Previous PPV23: n (%)	17 (61)	29 (88)	**0.02**[a]
Years from last PPV23	9.9 (5.0)	11.0 (4.2)	0.26[b]

4.3 Vaccination-related adverse events

There were no significant differences in the number of individuals reporting vaccination-related events or the total number of such events between patients with CKD and controls in this study. A total of 20 individuals (33%) reported adverse events within 7 days of vaccination (11 controls, 9 patients with CKD), with less than 10% reporting 2 or more events (Table 4-3). The majority of these were local injection site reactions including pain, redness and swelling, all of which had completely resolved by the second follow-up timepoint (day 28).

Table 4-3 Vaccination-related adverse events reported within 7 days.

N and (%) shown unless stated; [a]mid P 2-tailed p value; [b]Fisher's exact 2-tailed p value; significance defined as $p<0.05$.

	Controls n=28	CKD n=33	p value
Participants reporting any vaccination-related adverse events: n (%)	11 (39)	9 (27)	0.41[b]
Participants reporting ≥2 vaccination-related adverse events: n (%)	4 (14)	2 (6)	0.40[b]
Total number of vaccination-related events reported within 7 days	**15**	**12**	0.32[a]
Injection site reactions	10 (67)	7 (58)	0.80[a]
Malaise/fatigue	2 (13)	1 (8)	0.75[a]
Flu-like symptoms	2 (13)	1 (8)	0.75[a]
Fever	0 (0)	1 (8)	0.44[a]
Arthralgia/myalgia	0 (0)	2 (17)	0.20[a]
Headache	1 (7)	0 (0)	0.56[a]

4.4 Humoral responses to TIV antigens are similar between older patients with CKD and controls

To systematically interrogate the humoral response to TIV, I first evaluated pre-vaccination (baseline) strain specific influenza HAI titres. Due to heterogeneity in the composition of TIV across the 3 seasons of this study, I initially examined each season in turn. At baseline, the only difference seen between the patient groups was a significantly higher HAI GMT to the A/H3N2/Switzerland strain in patients with CKD than controls in the 2015-2016 vaccination season (Table 4-4). However, significance was lost when previous exposure to the antigen (e.g. through the previous year's TIV) was taken into account (p=0.07).

Table 4-4 Baseline influenza HAI GMTs – study participants split by disease group and vaccination season.

Geometric mean titres and 95% confidence intervals shown. Unpaired t-test on log-transformed data 2-tailed p value shown, $p<0.05$ considered significant and significant results highlighted in bold.

	Controls	CKD	p value
2015-2016 season	**n=10**	**n=13**	
A/H1N1/California	20.8 (10.1-42.9)	25.0 (12.5-50.1)	0.70
A/H3N2/Switzerland	9.3 (5.1-16.9)	24.8 (13.6-45.2)	***0.02***
B/Phuket	5.0 (5.0 -5.0)	6.2 (4.5-8.5)	0.21
2016-2017 season	**n=9**	**n=10**	
A/H1N1/California	23.6 (9.9-56.2)	18.9 (7.5-47.4)	0.69
A/H3N2/Hong Kong	85.8 (33.5-219.5)	72.5 (33.8-155.5)	0.75
B/Brisbane	10.0 (4.5-22.2)	6.4 (4.6-8.9)	0.24
2017-2018 season	**n=9**	**n=10**	
A/H1N1/Michigan	20.9 (10.3-42.7)	14.7 (6.3-34.5)	0.49
A/H3N2/Hong Kong	189.1 (66.6-536.7)	95.7 (28.9-317.0)	0.35
B/Brisbane	18.8 (7.0-50.6)	12.5 (6.2-25.1)	0.44

Patients with CKD and age matched controls had similar baseline strain-specific HAI GMTs prior to first and repeat exposures to vaccine influenza strains (Table 4-5 and Table 4-6, respectively).

Table 4-5 Influenza HAI GMTs for first exposure to listed strains – study participants split by disease group.

ARR: geometric mean of antibody response ratio, AMR: geometric mean of antibody maintenance ratio. * - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 0 and Day 28 titres within controls/patients with CKD; ~ - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 28 and Month 6 titres within controls/patients with CKD; # - p<0.05 2-tailed paired t test on log transformed data: comparison of Month 6 and Day 0 titres within controls/patients with CKD.

Influenza strain	Timepoint	Controls		CKD		Unpaired t test on transformed data
		n	GMT (95% CI)	n	GMT (95% CI)	
H1N1/Michigan	Day 0	9	20.9 (10.3-42.7)	11	18.3 (7.4-45.0)	0.80
	Day 28	9	54.4 (21.5-137.6)*	11	38.0 (17.0-84.8)*	0.51
	Month 6	8	29.8 (9.8-90.4)~	10	13.7 (6.3-30.1)~	0.20
	ARR		**2.6 (1.3-5.1)**		**2.1 (1.4-3.2)**	**0.51**
	AMR		**0.53 (0.33-0.83)**		**0.45 (0.29-0.69)**	**0.56**
H3N2/Hong Kong	Day 0	10	74.2 (30.5-180.2)	11	68.7 (34.5-137.0)	0.88
	Day 28	10	127.8 (56.3-290.2)*	11	86.1 (37.2-200.0)	0.46
	Month 6	10	136.9 (65.2-287.7)	9	86.4 (29.3-254.5)	0.42
	ARR		**1.7 (1.0-2.9)**		**1.3 (0.8-1.9)**	**0.29**
	AMR		**1.1 (0.71-1.6)**		**0.96 (0.64-1.3)**	**0.64**
B/Brisbane	Day 0	10	9.3 (4.5-19.1)	11	6.3 (4.6-8.5)	0.25
	Day 28	10	14.1 (6.2-32.1)	11	11.6 (5.6-24.0)	0.69
	Month 6	10	10.7 (5.2-22.0)	9	9.3 (4.3-20.1)~	0.76
	ARR		**1.5 (0.9-2.6)**		**1.8 (1.0-3.5)**	**0.61**
	AMR		**0.76 (0.47-1.2)**		**0.81 (0.66-0.99)**	**0.79**
B/Phuket	Day 0	10	5.0 (5.0-5.0)	13	6.2 (4.5-8.5)	0.21
	Day 28	10	11.6 (5.7-23.6)*	13	9.3 (5.2-16.4)	0.58
	Month 6	10	7.6 (4.7-12.2)~	12	7.1 (4.2-12.2)	0.86
	ARR		**2.3 (1.1-4.7)**		**1.5 (1.0-2.3)**	**0.22**
	AMR		**0.65 (0.49-0.87)**		**0.73 (0.47-1.1)**	**0.64**

Table 4-6 Influenza HAI GMTs for repeat exposure to listed strains – study participants split by disease group.

ARR: geometric mean of antibody response ratio, AMR: geometric mean of antibody maintenance ratio. * - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 0 and Day 28 titres within controls/patients with CKD; ~ - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 28 and Month 6 titres within controls/patients with CKD; # - p<0.05 2-tailed paired t test on log transformed data: comparison of Month 6 and Day 0 titres within controls/patients with CKD.

Influenza strain	Timepoint	Controls		CKD		Unpaired t test on transformed data
		n	GMT (95% CI)	n	GMT (95% CI)	
H1N1/California	Day 0	18	24.0 (14.6-40.0)	21	25.5 (15.1-43.0)	0.87
	Day 28	18	48.9 (35.8-83.9)*	21	54.4 (29.1-101.7)*	0.99
	Month 6	18	39.4 (26.5-58.5)~#	20	32.9 (16.6-65.0)~	0.64
	ARR		**2.3 (1.5-3.4)**		**2.1 (1.5-3.1)**	**0.80**
	AMR		**0.71 (0.55-0.93)**		**0.66 (0.45-0.97)**	**0.71**
H3N2/Switzerland	Day 0	8	9.6 (4.5-20.7)	12	22.5 (12.1-41.7)	0.07
	Day 28	8	47.6 (24.2-93.4)*	12	80.0 (31.9-200.7)*	0.37
	Month 6	8	28.3 (12.4-64.2)~#	11	45.9 (22.0-95.6)~#	0.34
	ARR		**4.3 (2.4-7.7)**		**3.6 (1.6-7.7)**	**0.50**
	AMR		**0.59 (0.39-0.90)**		**0.57 (0.33-1.0)**	**0.91**
H3N2/Hong Kong	Day 0	9	250.4 (96.7-648.2)	9	105.4 (27.6-403.4)	0.25
	Day 28	9	324.7 (135.1-780.4)	9	155.0 (52.5-457.2)	0.24
	Month 6	7	241.8 (76.0-768.9)~	9	120.7 (39.5-369.0)	0.33
	ARR		**1.3 (0.8-2.2)**		**1.5 (1.0-2.2)**	**0.66**
	AMR		**0.67 (0.46-0.98)**		**0.78 (0.55-1.1)**	**0.52**
B/Brisbane	Day 0	8	22.1 (7.6-64.3)	9	13.8 (6.5-29.4)	0.40
	Day 28	8	26.3 (8.8-78.6)	9	17.4 (7.6-39.8)*	0.48
	Month 6	7	24.8 (6.4-95.4)	8	18.0 (7.7-41.9)	0.62
	ARR		**1.2 (0.9-1.5)**		**1.3 (1.3-1.5)**	**0.67**
	AMR		**0.91 (0.71-1.2)**		**0.88 (0.77-1.0)**	**0.84**

Baseline HAI GMT against A/H3N2 strains correlated significantly with baseline titres to influenza B strains (Pearson R: 0.48, p<0.001), but not A/H1N1 strains. Increasing age was also significantly associated with higher baseline HAI GMT to A/H3N2 (Pearson R:

0.37, p=0.004) and B strains (Pearson R: 0.31, p=0.02), but not A/H1N1. There were no significant relationships between baseline HAI GMTs to any influenza vaccine strains and measures of renal disease (eGFR, ACR), multimorbidity (CCI, medication burden), glycaemic control (HbA1c), latent CMV infection (CMV-specific IgG titre) or inflammation (hsCRP).

In order to compare vaccine-induced humoral responses in a "like-for-like" manner, first and repeat exposures to different influenza strains were, in the first instance, analysed separately. Table 4-5 and Table 4-6 summarise HAI GMTs and 95% confidence intervals at day 0, day 28 and month 6 study timepoints, together with the statistical differences within this data.

Both patient groups generally increased HAI titres following 1st exposure to A/H1N1/California and A/H3N2/Switzerland. However, subject numbers were too small for meaningful statistical comparisons (maximum n=2), so these data are excluded from subsequent analysis. Controls and patients with CKD were able to significantly increase strain-specific HAI titres between day 0 and day 28 post-vaccination upon first or repeat exposure to A/H1N1 strains (Table 4-5, Table 4-6, Figure 4-1 A,E and Figure 4-2 A,E). The response to A/H3N2 strains was more variable. On first exposure to the A/H3N2/Hong Kong vaccine antigen, controls had a significant increase in strain-specific HAI titres, but patients with CKD did not (Figure 4-1 B,F). However, both controls and patients with CKD had a robust increase in HAI titres after repeat exposure to A/H3N2/Switzerland (Figure 4-2 B,F), but not A/H3N2/Hong Kong (Figure 4-2 C,G). This is likely to reflect some differences in immunogenicity of Switzerland versus Hong Kong strains of influenza A/H3N2 and also the small sample size in this like-for-like analysis.

Figure 4-1 Primary response to TIV antigens.

Strain-specific HAI titres shown at baseline and day 28 after vaccination for individual controls (A-D) and patients with CKD (E-H). * $p<0.05$ – paired t-test on log transformed titres.

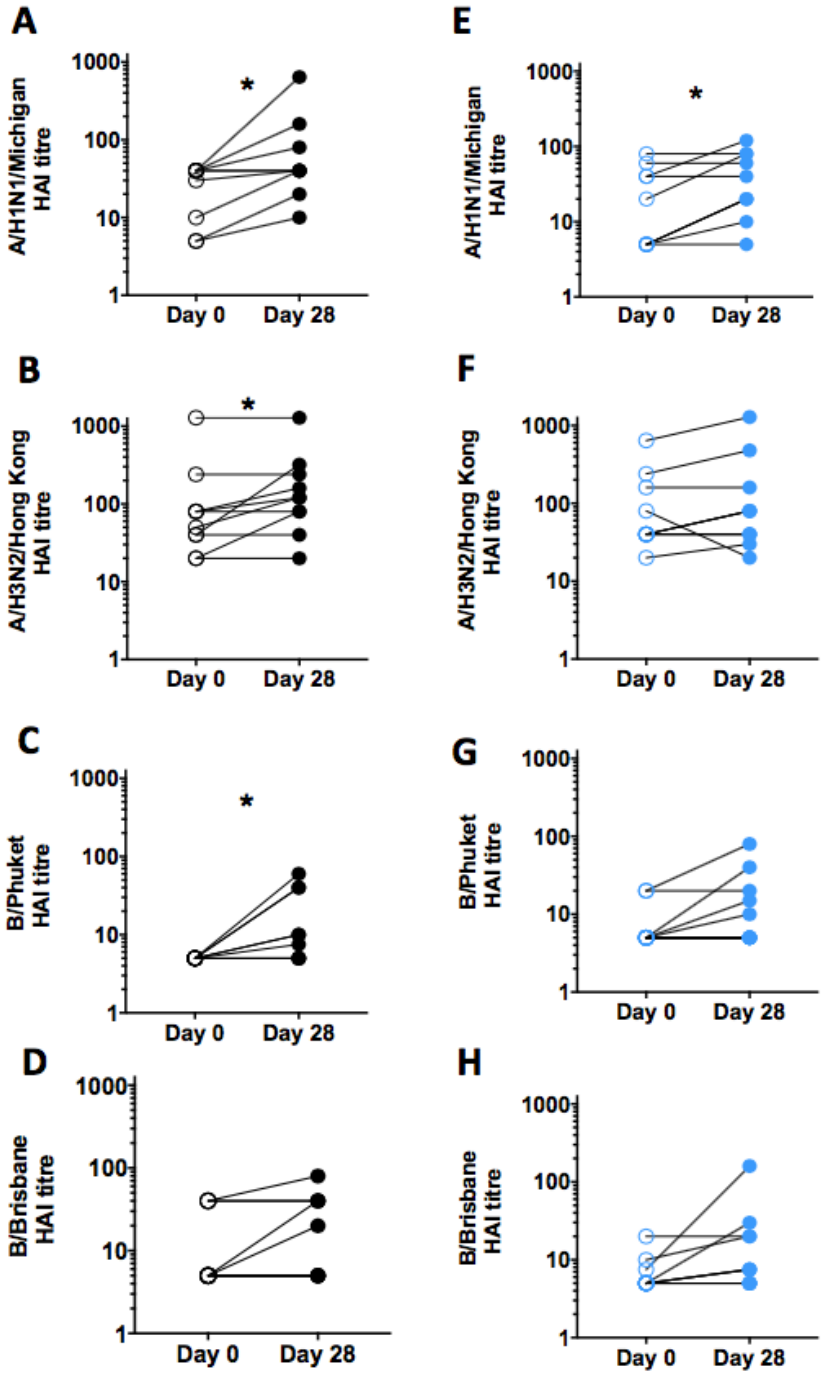
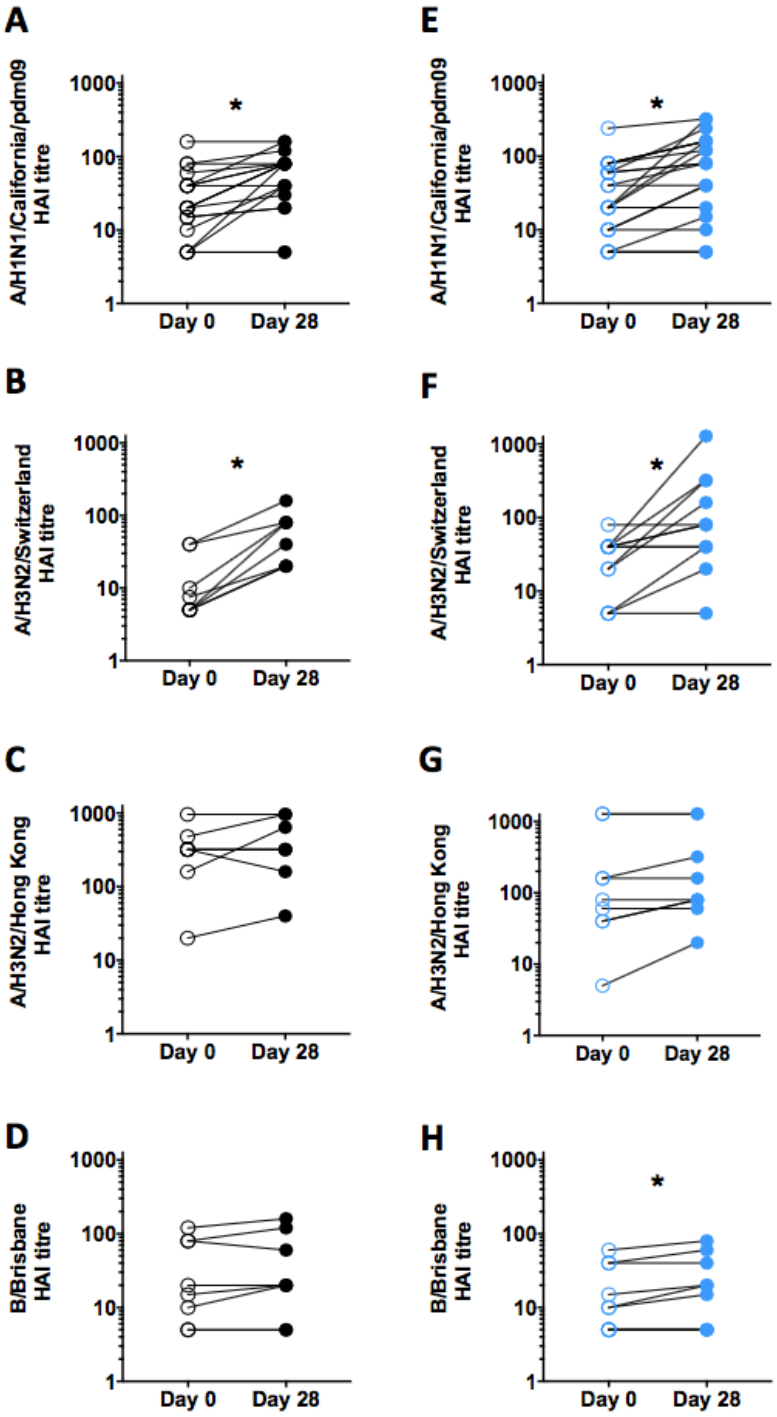


Figure 4-2 Secondary response to TIV antigens.

Strain-specific HAI titres shown at baseline and day 28 after vaccination for individual controls (A-D) and patients with CKD (E-H). * $p<0.05$ – paired t-test on log transformed titres.



Responses to B strains were generally poor in all study participants. In controls, a significant increase in HAI titres post-vaccination was seen only on first exposure to B/Phuket (Figure 4-1 C) and not on first or repeat exposure to B/Brisbane (Figure 4-1 D, Figure 4-2 D). Patients with CKD generally increased titres after first exposure to both B/Phuket and B/Brisbane (Figure 4-1 G, H), but this fell short of statistical significance (paired t-test on log transformed data 2-tailed p=0.06). Repeat exposure to B/Brisbane, however, did yield significant increases in HAI titre in patients with CKD (Figure 4-2 H).

The magnitude of humoral responses to individual influenza strains, as measured by the antibody response ratio (geometric mean of Day 28/Baseline titre; ARR), was greatest for first and repeat exposures to A/H1N1 strains and repeat exposure to A/H3N2/Switzerland (Table 4-5 and Table 4-6). The ARR was generally lower in patients with CKD than controls on first or repeat exposure to most strains, but this did not reach statistical significance.

As there was little difference between patients with CKD and controls when individual influenza strains and previous exposures were considered separately, data were then combined to give representative results for responses to all strains of A/H1N1, A/H3N2 and B influenza (Table 4-7 summarises HAI GMTs, 95% confidence intervals and statistical differences within the data). This method also increased the sample size and hence, power, of statistical analysis.

Table 4-7 Influenza HAI responses by strain and disease group – combined data.

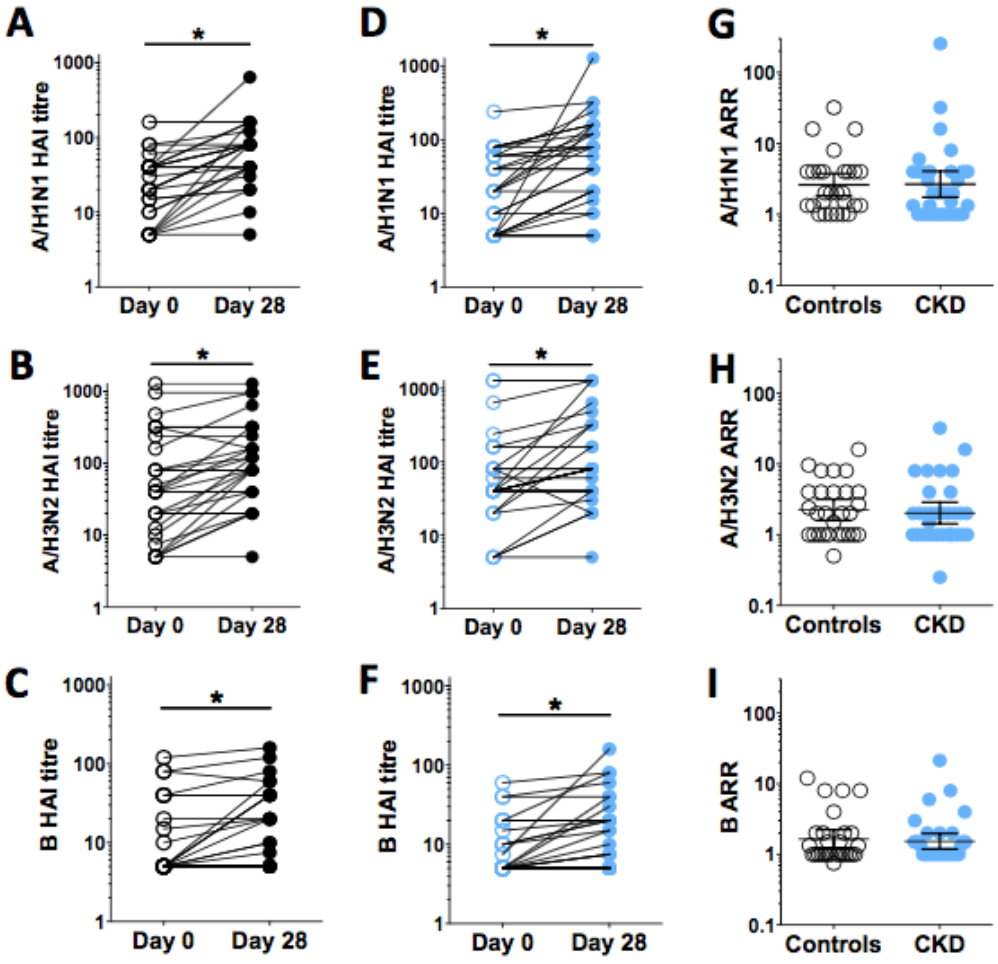
ARR: geometric mean of antibody response ratio, AMR: geometric mean of antibody maintenance ratio. * - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 0 and Day 28 titres within controls/patients with CKD; ~ - p<0.05 2-tailed paired t test on log transformed data: comparison of Day 28 and Month 6 titres within controls/patients with CKD; # - p<0.05 2-tailed paired t test on log transformed data: comparison of Month 6 and Day 0 titres within controls/patients with CKD.

Influenza strain	Timepoint	Controls		CKD		Unpaired t test on transformed data
		n	GMT (95% CI)	n	GMT (95% CI)	
A/H1N1	Day 0	28	21.7 (14.8-31.9)	33	19.6 (12.8-29.8)	0.79
	Day 28	28	56.8 (39.0-82.6)*	33	52.0 (31.8-85.0)*	0.85
	Month 6	27	37.2 (25.4-54.5)~#	30	24.6 (14.6-41.4)~	0.73
	ARR		**2.6 (1.8-3.8)**		**2.7 (1.7-4.1)**	**0.95**
	AMR		**0.65 (0.52-0.80)**		**0.58 (0.43-0.77)**	**0.54**
A/H3N2	Day 0	28	50.0 (25.6-97.4)	33	51.7 (31.9-83.7)	0.58
	Day 28	28	111.8 (66.3-188.3)*	33	104.6 (64.2-170.3)*	0.61
	Month 6	27	81.9 (46.0-146.0)~#	30	77.3 (47.3-126.3)~#	0.76
	ARR		**2.2 (1.6-3.2)**		**2.0 (1.4-2.9)**	**0.88**
	AMR		**0.74 (0.58-0.95)**		**0.71 (0.56-0.91)**	**0.81**
B	Day 0	28	9.6 (6.3-14.4)	33	7.7 (6.0-10.0)	0.57
	Day 28	28	15.8 (10.1-24.6)*	33	11.9 (8.2-17.1)*	0.59
	Month 6	27	11.7 (7.6-18.1)~#	30	9.8 (6.8-14.1)~#	0.73
	ARR		**1.6 (1.2-2.2)**		**1.5 (1.2-2.0)**	**0.95**
	AMR		**0.75 (0.62-0.91)**		**0.80 (0.68-0.95)**	**0.61**

Overall, both controls and patients with CKD significantly increased HAI titres to all TIV strains at day 28 post-vaccination and by an equivalent magnitude (Table 4-7 and Figure 4-3).

Figure 4-3 Summary of humoral response to TIV antigens- comparison between controls and patients with CKD.

Strain-specific HAI titres shown at baseline and day 28 after vaccination for individual controls (A-C) and patients with CKD (D-F), together with strain-specific antibody response ratios (ARR) – panels G-I. *p<0.05 – paired t-test on log transformed titres. Error bars show geometric mean and 95% CI.



Influenza A strains (in particular H1N1) elicited a greater magnitude of HAI titre increase at day 28 than B strains. Increases in influenza A strain ARR were significantly associated with lower baseline HAI titres (Pearson R: -0.38, p=0.002 for A/H1N1 strains; R: -0.48, p<0.0001 for A/H3N2 strains). This was not the case for influenza B strains. However, as

seen with baseline HAI titres, higher A/H3N2 strain ARR was significantly associated with higher ARR to B strains (Pearson R: 0.30, p=0.02).

Increasing age was significantly associated with lower ARR to A/H1N1 (Pearson R: -0.35, p=0.006) and A/H3N2 strains (Pearson R: -0.32, p=0.01) and approached significance for influenza B strains (Pearson R: -0.22, p=0.09). However, as seen with baseline HAI titres, there were no significant associations between ARR to any of the vaccine influenza strains or measures of renal disease (eGFR, ACR), multimorbidity (CCI, medication burden), glycaemic control (HbA1c), latent CMV infection (CMV-specific IgG titre) or inflammation (hsCRP).

Older age (p=0.02) and higher baseline HAI titres against A/H1N1 strains (p=0.004) were significant predictors of lower A/H1N1 ARR in a linear regression model that also included gender, CKD status and CMV serostatus. Higher baseline A/H3N2 HAI titres (p=0.001) also emerged as a significant predictor of lower A/H3N2 ARR when the same model was applied to this vaccine strain. However, no significant predictors of influenza B strain ARR were identified on multivariate analysis.

In summary, influenza A strains (particularly H1N1) elicited a greater increase in neutralising strain-specific HA antibody after vaccination with TIV than was seen for B strains in this study of older adults with and without chronic disease. No differences in the absolute HAI GMTs or the magnitude of HAI titre increase post-vaccination (ARR) was detected when patients with CKD were compared with age-matched controls. Only age and pre-vaccination strain-specific HAI titres emerged as significant predictors of the magnitude of influenza A strain HAI increase at day 28 post-vaccination (ARR).

4.5 Maintenance of TIV response

When primary and secondary responses to different influenza strains were analysed separately, significant reductions in strain-specific HAI titres at month 6 post-vaccination were seen in both controls and patients with CKD following primary and secondary exposures to A/H1N1 strains and repeat exposure to A/H3N2/Switzerland (summarised in Table 4-5 and Table 4-6, data also shown in Figure 4-4 A,E and Figure 4-5 A,B,E and F). No significant reductions in HAI titre from levels reached at day 28 were seen at month 6 following first exposure to A/H3N2/Hong Kong in either controls or patients with CKD (Figure 4-4 B,F), but controls exposed to this antigen a second time did demonstrate a significant reduction in titre from day 28 to month 6 after vaccination (Figure 4-5 C). The patterns seen with influenza B antigens were variable, with significant reductions in titre at month 6 observed in controls only following first exposure to B/Phuket (Figure 4-4 C) and patients with CKD only following first exposure to B/Brisbane (Figure 4-4 H).

Figure 4-4 Maintenance of responses following first exposure to TIV antigens.

Strain-specific HAI titres shown at day 28 and month 6 after vaccination for individual controls (A-D) and patients with CKD (E-H). * $p<0.05$ – paired t-test on log transformed titres.

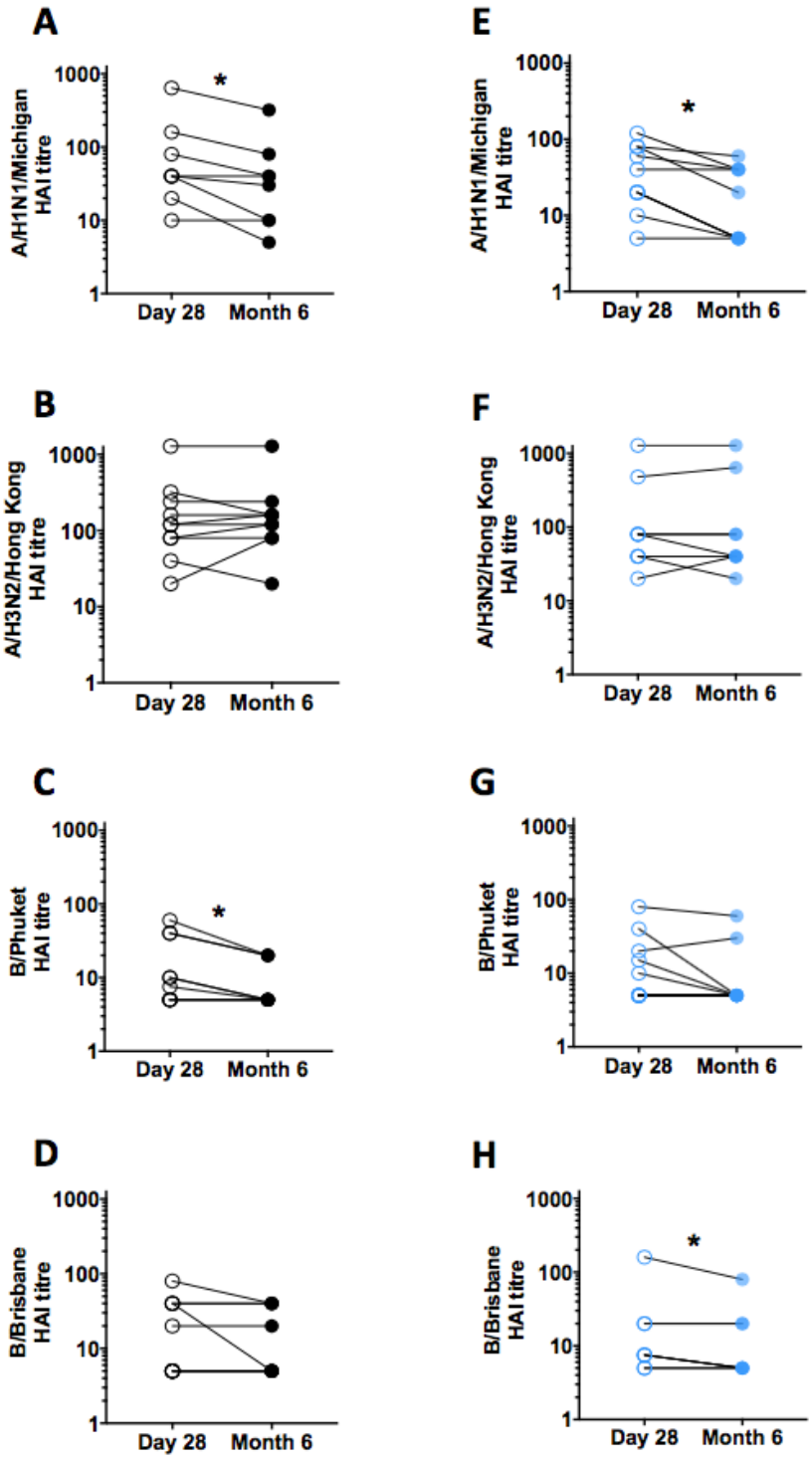
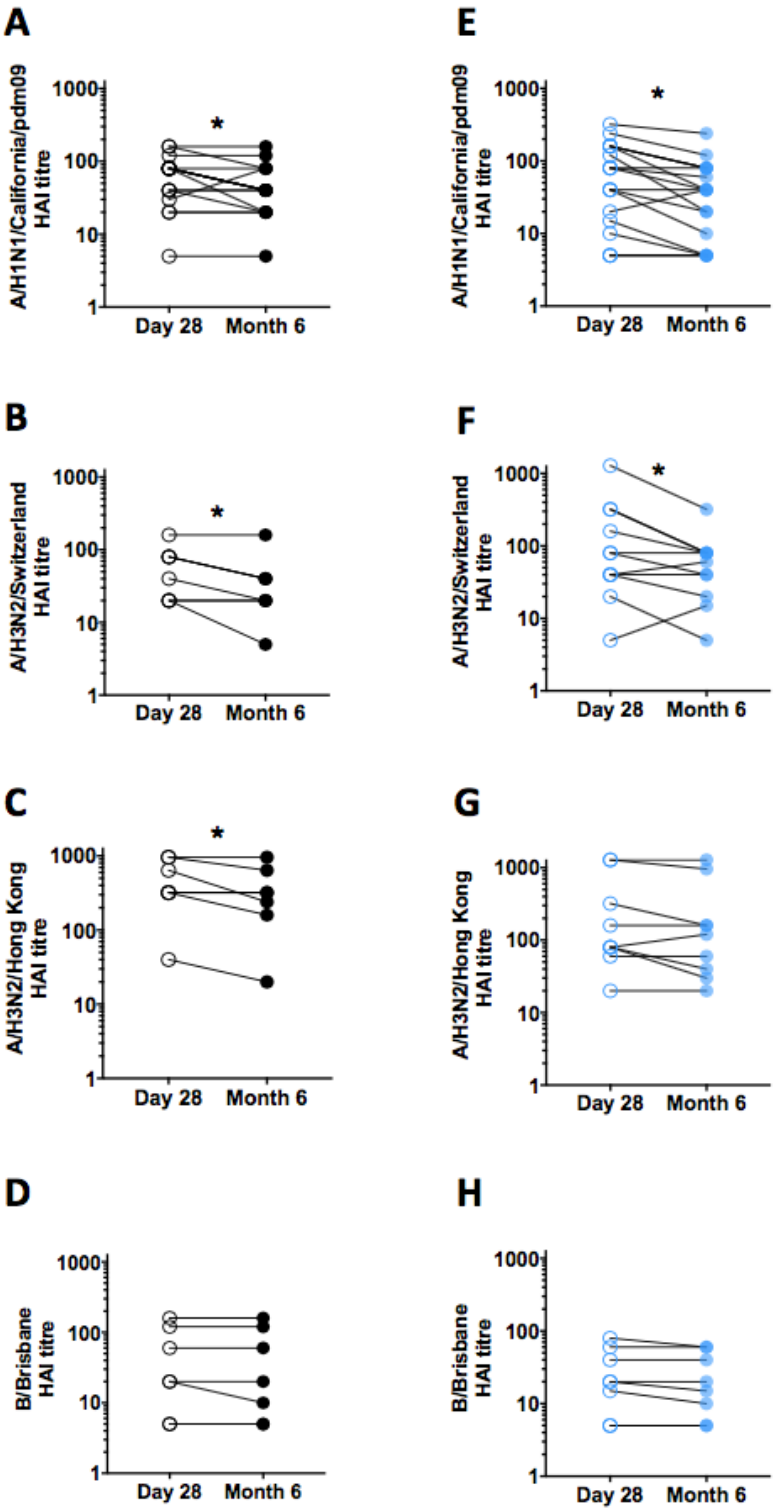


Figure 4-5 Maintenance of responses following repeat exposure to TIV antigens.

Strain-specific HAI titres shown at day 28 and month 6 after vaccination for individual controls (A-D) and patients with CKD (E-H). * $p<0.05$ – paired t-test on log transformed titres.



Interestingly, the humoral response following first exposure to influenza A strains and all exposures to influenza B strains appeared short lived. Month 6 HAI titres were significantly higher than pre-vaccination levels only following repeat exposure to A/H3N2/Switzerland in both controls and patients with CKD, and after repeat exposure to A/H1N1/California in controls (summarised in Table 4-5 and Table 4-6). First exposure to any influenza strains contained in TIV and all exposures to influenza B strains did not yield significantly different HAI titres at month 6 compared to pre-vaccination levels in either controls or patients with CKD.

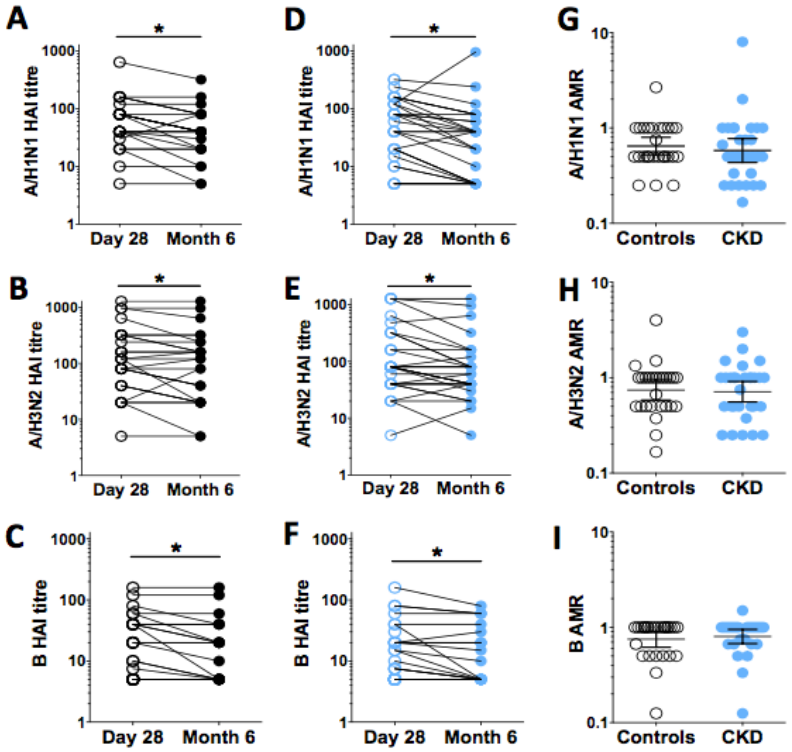
The magnitude of strain-specific HAI titre change at month 6 from peak titres at day 28 was assessed using the geometric mean antibody maintenance ratio (month 6/day 28 titre; AMR), where a value of 1 represents maintenance of titre and values less than 1 represent a reduction. Strain-specific AMRs were similar between controls and patients with CKD for both first and repeat exposures to vaccine antigens.

When data were combined to give representative results for responses to all A/H1N1, A/H3N2 and B strains (summarised in Table 4-7 and Figure 4-6), both patient groups had significantly lower HAI GMTs at month 6 than day 28 post-vaccination to all 3 TIV strains. Interestingly, month 6 GMTs were significantly higher than pre-vaccination titres in controls for all TIV strains, but not in patients with CKD, where A/H1N1 titres at month 6 approximated pre-vaccination levels. Overall, strain-specific AMRs were similar between controls and patients with CKD (Table 4-7 and Figure 4-6). Higher strain-specific ARR was significantly associated with lower AMR for that strain (Pearson R: -0.54, $p<0.0001$ for A/H1N1; R= -0.68, $p<0.0001$ for A/H3N2 strains and R: -0.76, $p<0.0001$ for B strains). Strain-specific ARRs were significant predictors of AMR in a linear regression model that also included age, gender, CKD and CMV serostatus for each of the TIV

strains ($p<0.0001$). As such, this suggests that those individuals with greater change in HAI titre from baseline to peak are more likely to reduce titres by month 6 than those who do not mount as robust a response. This may simply reflect the short-lived nature of the response to TIV antigens, with greater "maintenance" of titre simply reflecting little change in HAI titre across the study for individuals with poor vaccine-induced responses.

Figure 4-6 Summary of maintenance of humoral response to TIV antigens- comparison between controls and patients with CKD.

Strain-specific HAI titres shown at day 28 and month 6 after vaccination for individual controls (A-C) and patients with CKD (D-F), together with strain-specific antibody maintenance ratios (AMR) – panels G-I. *$p<0.05$ – paired t-test on log transformed titres. Error bars show geometric mean and 95% CI.



In summary, older adults with and without chronic disease significantly reduce strain-specific HAI titres from peak (day 28) to month 6 after vaccination with TIV. Although titres at month 6 were significantly higher than pre-vaccination titres for most strains, the significant inverse correlations between strain-specific ARR and AMR suggest that robust responses to TIV are relatively short-lived. This supports the current recommendation for routine annual influenza vaccination in this patient population.

4.6 Clinical parameters of adequate TIV response

A post-vaccination (day 28 onwards) strain-specific HAI titre of 40 or above is associated with 50% protection against disease with that influenza strain on a population level and will henceforth be referred to as the "protective titre" (31). Controls and patients with CKD had equivalent proportions of individuals with protective HAI titres against all 3 influenza strains at baseline and day 28 post-vaccination (Table 4-8). Healthy individuals with HAI of 40 or above prior to vaccination are expected to be able to mount a 4-fold increase in titres post-vaccination. An adequate response to influenza vaccination is, therefore, defined as the conversion from a non-protective to protective titre or a 4-fold increase from baseline if starting titres are 40 or above. This approach allows first and repeat exposures to influenza antigens to be considered together. Adequate response to TIV as a whole is defined here as an adequate response to 2 of 3 antigens contained in that season's vaccine.

Table 4-8 Adequate responses to seasonal TIV in SONIC study participants.

N and % shown. Adequate response was defined as the conversion strain-specific HAI titre of <40 pre-vaccination to HAI≥40 at day 28 or a 4-fold increase from baseline if starting titres are 40) – study participants are split by disease group and vaccination season.

Adequate response		**Controls**			**CKD**	
	≥40 at baseline	≥40 at Day 28	**Adequate response**	≥40 at baseline	≥40 at Day 28	**Adequate response**
2015-2016 season		n=10			n=13	
A/H1N1/California	4 (40)	8 (80)	**4 (40)**	5 (38)	11 (85)	**6 (46)**
A/H3N2/Switzerland	2 (20)	6 (60)	**5 (50)**	8 (62)	11 (85)	**6 (46)**
B/Phuket	0 (0)	3 (30)	**3 (30)**	0 (0)	2 (15)	**2 (15)**
2/3 TIV	**1 (10)**	**6 (60)**	**4 (40)**	**3 (23)**	**11 (85)**	**3 (23)**
2016-2017 season		n=9			n=10	
A/H1N1/California	4 (44)	7 (78)	**4 (44)**	5 (50)	6 (60)	**2 (20)**
A/H3N2/Hong Kong	8 (89)	8 (89)	**1 (11)**	9 (90)	9 (90)	**0 (0)**
B/Brisbane	3 (33)	4 (44)	**1 (11)**	0 (0)	1 (10)	**1 (10)**
2/3 TIV	**5 (56)**	**7 (78)**	**1 (11)**	**5 (50)**	**5 (50)**	**0 (0)**
2017-2018 season		n=9			n=10	
A/H1N1/Michigan	5 (56)	7 (78)	**4 (44)**	4 (40)	5 (50)	**1 (10)**
A/H3N2/Hong Kong	7 (78)	9 (100)	**3 (33)**	9 (90)	9 (90)	**0 (0)**
B/Brisbane	3 (33)	3 (33)	**0 (0)**	3 (30)	3 (30)	**0 (0)**
2/3 TIV	**5 (56)**	**6 (67)**	**2 (22)**	**4 (40)**	**4 (40)**	**0 (0)**
ALL SEASONS		n=28			n=33	
A/H1N1 strain	13 (46)	22 (79)	**12 (43)**	14 (42)	22 (67)	**9 (27)**
A/H3N2 strain	17 (61)	23 (82)	**9 (32)**	26 (79)	29 (88)	**6 (18)**
B strain	6 (21)	10 (36)	**4 (14)**	3 (9)	6 (18)	**3 (9)**
2/3 TIV	**11 (39)**	**20 (71)**	**7 (25)**	**12 (36)**	**21 (64)**	**4 (12)**

When responses to TIV as a whole were considered across all vaccination seasons, a surprisingly small proportion of all participants mounted an adequate response to the vaccine (less than 1 in 5) - Table 4-8. Patients with CKD generally performed worse than controls, with a lower proportion of individuals mounting an adequate response to 2 of 3 vaccine antigens (12% versus 25%, Fisher's exact 2-tailed p=0.32). The H1N1 influenza A strains appeared to be the most immunogenic overall using these clinical definitions (12

(43%) controls and 9 (27%) patients with CKD mounting adequate response, Fisher's exact 2-tailed p=0.28) and the B strains were the least (4 (14%) controls and 3 (9%) patients with CKD mounting adequate response, Fisher's exact 2-tailed p=0.69).

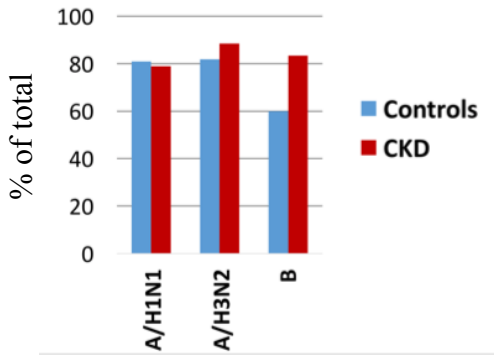
When vaccination seasons were considered separately, patients with CKD in the 2015-16 cohort had slightly greater protection to A/H3N2/Switzerland than controls at baseline (n=2 (20%) versus n=8 (62%) respectively, Fisher's exact p=0.09) mirroring the pattern seen for absolute HAI titres. However, the number and proportion of individuals achieving an adequate response to this antigen were similar between the groups (n=5 (50%) in controls and n=6 (46%) in patients with CKD).

Individuals that achieved protective HAI titres for any of the TIV strains at day 28 tended to maintain them at month 6 (Figure 4-7) and there were no significant differences between controls and patients with CKD. Overall, 80% (n=16) of controls maintained protective HAI titres to 2 of 3 TIV strains, compared with 67% (n=14) of patients with CKD (Fisher's exact 2-tailed p=0.48).

Overall, using clinical parameters of TIV humoral response, both patients with CKD and controls had poor responses to the vaccine as a whole, with less than 20% achieving adequate responses to 2 of 3 TIV antigens. Patients with CKD seemed to perform slightly worse compared to controls, but this did not reach statistical significance (possibly limited by sample size).

Figure 4-7 Proportion of individuals that maintained protective HAI titres (40 or more) at month 6 by TIV strain.

Blue – controls, red – patients with CKD.



4.7 Demographic and clinical characteristics of TIV responders

Due to the small number of TIV "responders" and lack of significant differences in TIV responses between controls and patients with CKD, all study participants were considered together in the following comparison of "responders" (n=11) and "non-responders" (n=50). Unfortunately, the number of "responder" individuals was too small for meaningful multivariate predictive analysis using logistic regression.

TIV responders were significantly younger (Table 4-9 and Figure 4-8 A), but with an approximately equal gender split, equivalent multimorbidity as measured by CCI and medication burden, smoking/alcohol exposure and prevalence of latent CMV (Table 4-9).

Table 4-9 Clinical characteristics of TIV responders versus non-responders.

N and % shown. *and blue highlighting denotes unpaired t test on transformed data 2-tailed $p<0.05$.

	Responder n=11	Non-responder n=50
Age, years	69*(6)	76*(11)
Male: n (%)	9 (82)	29 (58)
Smoking, pack year history	0 (20)	3 (16)
Current drinker: n (%)	9 (82)	36 (72)
Weekly alcohol intake of current drinkers, units	10 (9)	6 (18)
Charlson comorbidity index	2 (3)	3 (4)
Number of medications	3 (5)	6 (8)
DM: n (%)	4 (36)	16 (32)
CMV seropositive: n (%)	7 (64)	37 (74)
Participants reporting any infections: n (%)	7 (64)	16 (32)
Participants reporting respiratory infections: n (%)	5 (45)	13 (26)
Mortality: n (%)	0 (0)	3 (6)

CMV-specific IgG titre was equivalent between CMV seropositive responders and non-responders (Table 4-10). TIV responders generally had lower hsCRP and higher eGFR (Figure 4-8 B,C), but no significant differences were seen in any other biochemistry parameters, including HbA1c (also not altered when only individuals with DM were compared) and albumin (Table 4-10). No significant differences were seen between responders and non-responders in total white cell count, neutrophil count or NLR, however, Hb and lymphocyte counts seemed to be higher in CKD responders compared to non-responders (Figure 4-8 E,F). No differences were seen in levels of total IgA/M/G between responders and non-responders.

Figure 4-8 Clinical and laboratory parameters of TIV responders (R) versus non-responders (NR).

Age (A), hsCRP (B), eGFR (C & D), Hb (E) and lymphocyte count (F) shown. Teal colour represents TIV responders. Error bars show median +/- IQR. Dashed red lines in panel F represent limits of normal range. *unpaired t test on transformed data 2-tailed $p<0.05$; p values also shown for relationships approaching significance.

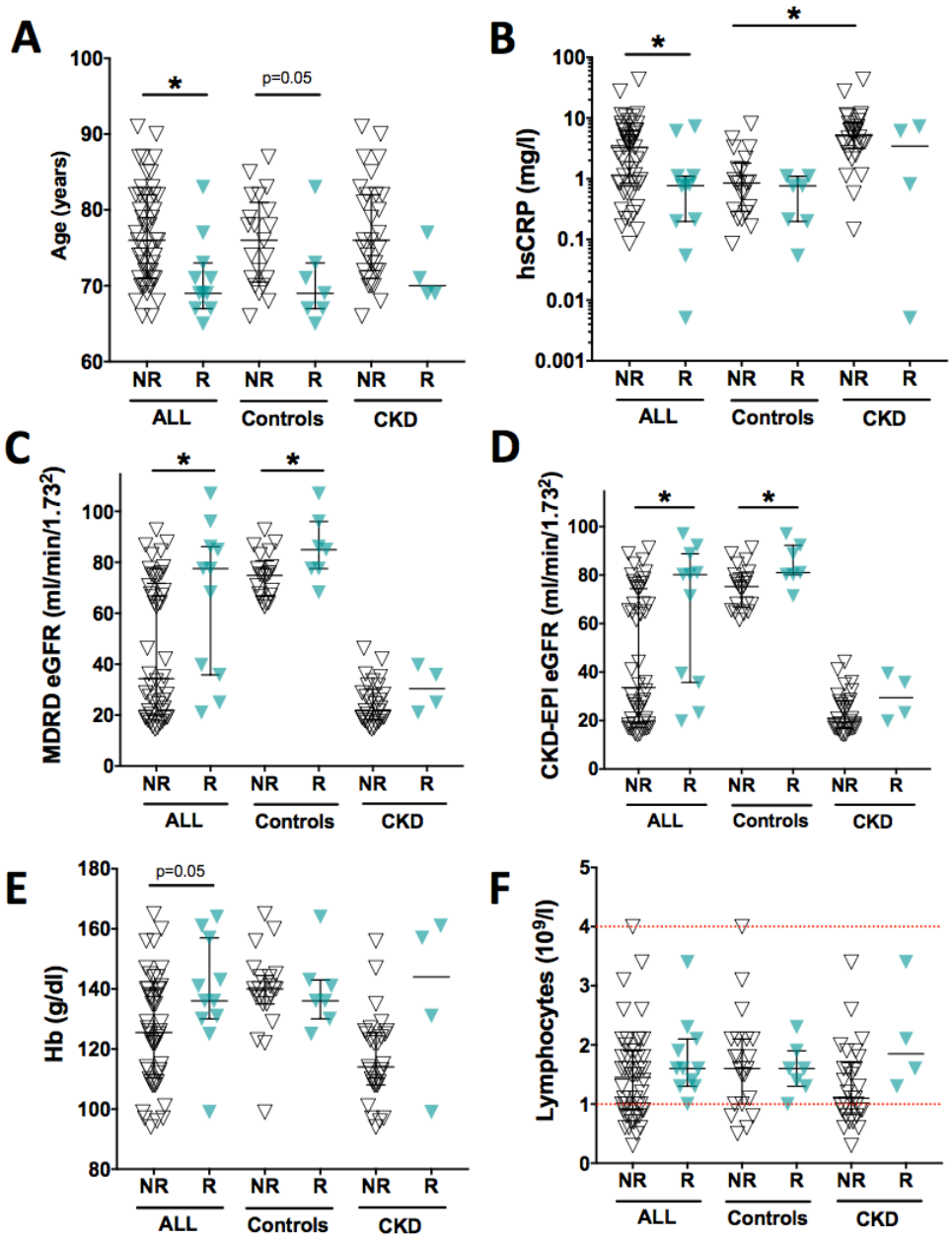


Table 4-10 Clinical laboratory characteristics of TIV responders versus non-responders. Median and IQR shown. * and blue colour denote unpaired t test on transformed data 2-tailed $p<0.05$.

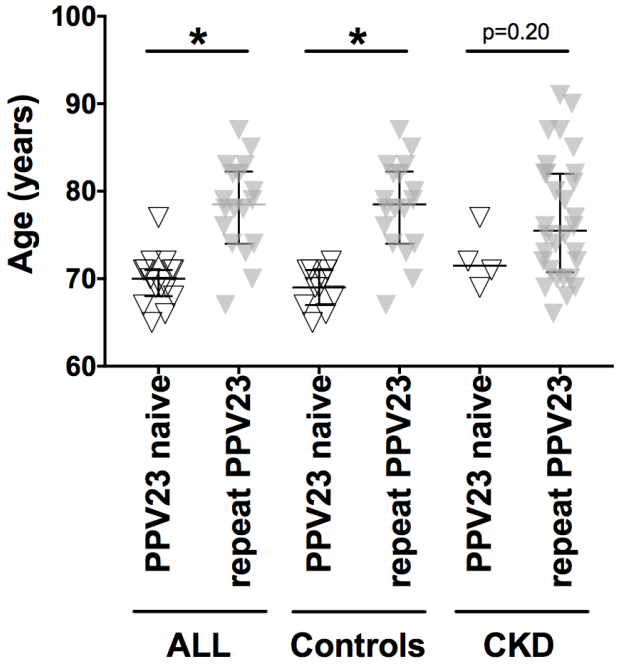
	Responder n=11	Non-responder n=50
Hb (g/dl)	136 (27)	126 (29)
WCC	6.5 (4.0)	6.4 (2.7)
neutrophils	4.8 (3.4)	3.9 (1.8)
lymphocytes	1.6 (0.8)	1.5 (1.0)
NLR	2.2 (2.9)	3.0 (2.4)
platelets	208 (74)	204 (66)
eGFR (MDRD)	**77.5*(50.3)**	**34.3*(51.6)**
eGFR (CKD-EPI)	**80.2*(53.1)**	**33.6*(55.6)**
ACR	1.0 (21.7)	6.4 (90.7)
hsCRP	**0.77*(0.90)**	**3.29*(5.34)**
HbA1c	40 (13)	41 (15)
Albumin	46 (5)	45 (4)
Total protein	72 (6)	72 (8)
Alkaline phosphatase	82 (29)	75 (29)
Calcium	2.40 (0.13)	2.41 (0.17)
Phosphate	1.05 (0.27)	1.15 (0.25)
Ferritin	101 (95)	126 (113)
Iron	17.0 (9.8)	14.3 (7.7)
Folate	6.3 (5.1)	8.0 (9.2)
Vitamin B12	371 (144)	398 (259)
IgA	1.9 (1.6)	2.2 (1.6)
IgG	11.2 (5.6)	10.5 (4.5)
IgM	1.0 (1.4)	0.6 (0.4)
CMV-specific IgG (CMV seropositive only)	153 (264)	137 (199)

4.8 Pre-vaccination titres of pneumococcal serotype-specific IgG are similar in older adults with CKD and controls

At baseline, patients with CKD had significantly greater coverage with PPV23 than controls, but duration from previous vaccination was similar, with a median of approximately 10 years (Table 4-2). Although controls and patients with CKD were matched for age across the whole study, those that had received a previous PPV23 vaccine were significantly older (median 69.5 years for vaccine naïve (IQR 3.8) and 78 years for revaccinees (IQR 9.3), Figure 4-9). This is expected, given contemporaneous guidelines for PPV23 to be given routinely at age 65.

Figure 4-9 Comparison of age between PPV23 naïve individuals and those receiving repeat PPV23 vaccination.

Grey symbols represent data from revaccinees. Error bars show median +/- IQR. *unpaired t test $p<0.05$.



No significant differences were seen in baseline serotype-specific anti-PnPS IgG titres between controls and patients with CKD (Table 4-11).

When controls and patients with CKD were split by previous vaccination status, CKD revaccinees had a trend for lower baseline titres against serotype 3 compared to controls (unpaired 2-tailed t-test on log transformed data p=0.10), which may be a chance finding, but could also suggest poorer maintenance of Pn3-specific long-term humoral immunity in CKD. Previously vaccinated controls and patients with CKD had significantly higher titres against serotype 4 than vaccine naïve individuals (unpaired t-test 2-tailed p=0.02 on log-transformed titres; geometric mean concentrations (GMCs) 0.06μg/ml (95% CI 0.03-0.11) and 0.17μg/ml (95% CI 0.11-0.27) respectively). This is expected as natural exposure to this serotype is infrequent (285, 286).

4.9 Patients with CKD and controls increase serotype-specific anti-PnPS IgG after vaccination with PPV23

GMCs of serotype-specific anti-PnPS before and at day 28 after PPV23 vaccination, together with statistical differences within the data, are summarised in Table 4-11. PPV23 vaccination significantly increased serotype-specific anti-PnPS IgG concentrations for all 12 serotypes tested from baseline to day 28 in both controls (Figure 4-10) and patients with CKD (Figure 4-11) – summarised in Figure 4-12 A.

Table 4-11 Serotype-specific anti-Pneumococcal IgG titres before and after PPV23 vaccination.

		Controls		CKD		p value
		n	GMC (95% CI)	n	GMC (95% CI)	
Pn1	Day 0	29	0.29 (0.14-0.59)	33	0.15 (0.08-0.28)	0.16
	Day 28	28	1.30*(0.63-2.65)	32	0.46*(0.24-0.90)	0.04
	Month 6	27	1.13~#(0.53-2.42)	29	0.35~#(0.17-0.74)	0.03
	ARR		**4.4 (2.8-7.1)**		**3.0 (1.9-4.7)**	**0.22**
	AMR		0.83 (0.72-0.96)		0.76 (0.67-0.86)	0.36
Pn3	Day 0	29	0.14 (0.08-0.24)	33	0.08 (0.05-0.13)	0.16
	Day 28	28	0.29*(0.15-0.54)	32	0.15*(0.09-0.26)	0.12
	Month 6	27	0.26#(0.14-0.48)	29	0.11~#(0.06-0.19)	0.04
	ARR		**2.3 (1.6-3.2)**		**1.9 (1.5-2.4)**	**0.38**
	AMR		0.98 (0.79-1.21)		0.75 (0.65-0.86)	0.03
Pn4	Day 0	29	0.15 (0.08-0.27)	33	0.12 (0.07-0.21)	0.57
	Day 28	28	0.39*(0.22-0.67)	32	0.24*(0.14-0.41)	0.21
	Month 6	27	0.35~#(0.19-0.62)	29	0.19~#(0.11-0.35)	0.16
	ARR		**2.7 (1.9-4.0)**		**2.0 (1.5-2.7)**	**0.17**
	AMR		0.84 (0.74-0.95)		0.78 (0.69-0.89)	0.45
Pn5	Day 0	29	0.63 (0.37-1.07)	33	0.48 (0.28-0.80)	0.44
	Day 28	28	1.70*(0.97-2.98)	32	0.93*(0.51-1.67)	0.14
	Month 6	27	1.83#(1.10-3.04)	29	0.69~#(0.38-1.25)	0.01
	ARR		**2.5 (1.7-3.7)**		**2.0 (1.4-2.8)**	**0.32**
	AMR		0.95 (0.83-1.08)		0.83 (0.72-0.96)	0.18
Pn6b	Day 0	29	0.29 (0.15-0.57)	33	0.44 (0.24-0.79)	0.35
	Day 28	28	0.86*(0.42-1.76)	32	0.77*(0.42-1.42)	0.80
	Month 6	27	0.69~#(0.32-1.46)	29	0.60~#(0.30-1.19)	0.79
	ARR		**3.0 (1.8-5.0)**		**1.9 (1.3-2.6)**	**0.09**
	AMR		0.81 (0.73-0.91)		0.81 (0.72-0.93)	0.98
Pn7f	Day 0	29	1.14 (0.69-1.87)	33	0.84 (0.54-1.30)	0.35
	Day 28	28	2.20*(1.41-3.44)	32	1.42*(0.92-2.19)	0.16
	Month 6	27	1.88~#(1.18-3.00)	29	1.30~#(0.80-2.12)	0.27
	ARR		**2.0 (1.5-2.7)**		**1.7 (1.3-2.3)**	**0.40**
	AMR		0.88 (0.80-0.98)		0.87 (0.77-0.98)	0.82
Pn9V	Day 0	29	0.61 (0.40-0.93)	33	0.51 (0.34-0.78)	0.55
	Day 28	28	1.19*(0.76-1.86)	32	0.82*(0.52-1.29)	0.24
	Month 6	27	1.15#(0.73-1.80)	29	0.72#(0.45-1.14)	0.14
	ARR		**2.0 (1.4-2.8)**		**1.6 (1.2-2.3)**	**0.43**
	AMR		0.95 (0.86-1.04)		0.88 (0.75-1.02)	0.40
Pn14	Day 0	29	2.22 (1.33-3.72)	33	2.40 (1.59-3.62)	0.81
	Day 28	28	3.92*(2.60-5.90)	32	3.33*(2.19-5.06)	0.57
	Month 6	27	3.85#(2.51-5.91)	29	3.00#(1.92-4.68)	0.41
	ARR		**1.8 (1.3-2.5)**		**1.5 (1.2-1.8)**	**0.19**
	AMR		0.95 (0.88-1.04)		0.92 (0.81-1.05)	0.64
Pn18c	Day 0	29	1.10 (0.66-1.84)	33	1.49 (0.89-2.51)	0.40
	Day 28	28	2.84*(1.71-4.72)	32	2.86*(1.70-4.80)	0.98
	Month 6	27	2.52#(1.46-4.36)	29	2.65#(1.47-4.78)	0.90
	ARR		**2.8 (1.9-4.2)**		**1.9 (1.5-2.5)**	**0.12**
	AMR		0.90 (0.80-1.00)		0.94 (0.87-1.03)	0.50
Pn19A	Day 0	29	0.68 (0.32-1.44)	33	0.62 (0.35-1.08)	0.82
	Day 28	28	1.48*(0.85-2.57)	32	1.05*(0.54-2.03)	0.43
	Month 6	27	1.59#(0.94-2.71)	29	0.99~#(0.51-1.93)	0.26
	ARR		**2.4 (1.4-4.0)**		**1.9 (1.4-2.4)**	**0.37**
	AMR		0.93 (0.84-1.03)		0.88 (0.78-0.99)	0.45
Pn19F	Day 0	29	0.99 (0.63-1.57)	33	0.92 (0.59-1.45)	0.82
	Day 28	28	2.49*(1.68-3.69)	32	1.56*(1.02-2.39)	0.11
	Month 6	27	2.36#(1.54-3.63)	29	1.28~#(0.79-2.08)	0.06
	ARR		**2.7 (1.8-4.1)**		**1.7 (1.3-2.2)**	**0.04**
	AMR		0.92 (0.82-1.03)		0.81 (0.72-0.92)	0.12
Pn23F	Day 0	29	0.60 (0.32-1.15)	33	0.34 (0.21-0.54)	0.14
	Day 28	28	1.86*(1.03-3.34)	32	0.67*(0.40-1.13)	0.01
	Month 6	27	1.43~#(0.78-2.60)	29	0.49~#(0.29-0.84)	0.008
	ARR		**3.4 (2.0-5.9)**		**2.0 (1.4-2.8)**	**0.09**
	AMR		0.81 (0.70-0.94)		0.71 (0.60-0.83)	0.20
PPV23	**ARR**		**3.6 (2.4-5.4)**		**2.4 (1.8-3.2)**	**0.09**
	AMR		0.93 (0.87-1.00)		0.86 (0.79-0.93)	0.12

Geometric mean concentrations (GMC) and 95% CI shown with unpaired t-test 2-tailed p value for log-transformed data.

ARR: antibody response ratio, AMR: antibody maintenance ratio.

*p<0.05 2-tailed paired t test on log transformed data: comparison of Day 0 and Day 28 titres;

~p<0.05 2-tailed paired t test on log transformed data: comparison of Day 28 and Month 6 titre;

#p<0.05 2-tailed paired t test on log transformed data: comparison of Month 6 and Day 0 titre.

Figure 4-10 Serotype-specific anti-PnPs IgG change from baseline to day 28 in individual control subjects.

Range of assay: 0.01 -10 microgram/m (dashed lines): all values below 0.01 were assigned a value of 0.01, all values above 10 were assigned a value of 10. Red line denotes WHO long-term protective threshold of 0.35microgram/ml. *denotes $p<0.05$ paired t-test performed on log-transformed data.

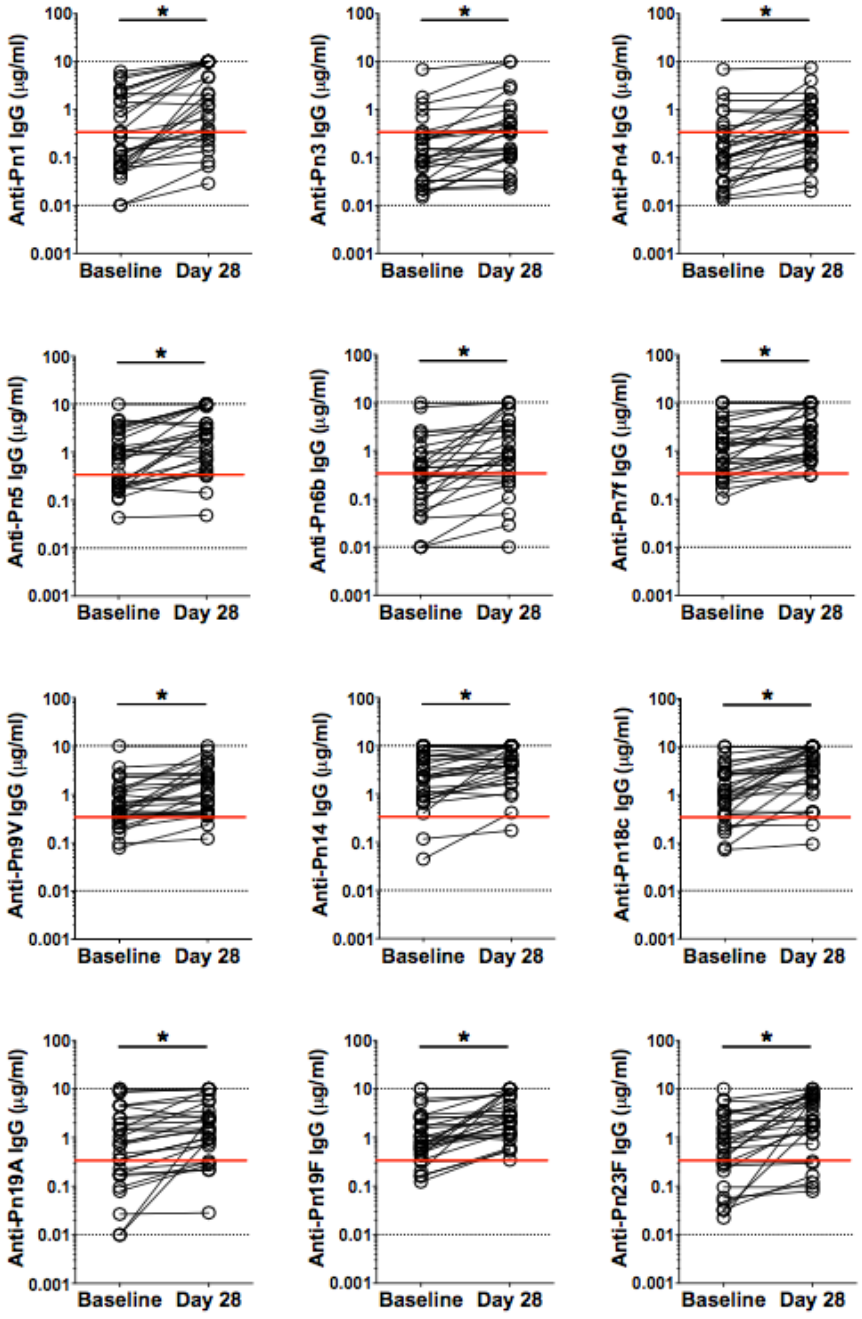


Figure 4-11 Serotype-specific anti-PnPs IgG change from baseline to day 28 in individual patients with CKD.

Range of assay: 0.01 -10 microgram/m (dashed lines): all values below 0.01 were assigned a value of 0.01, all values above 10 were assigned a value of 10. Red line denotes WHO long-term protective threshold of 0.35 microgram/ml. * denotes $p<0.05$ – paired t-test performed on log-transformed data.

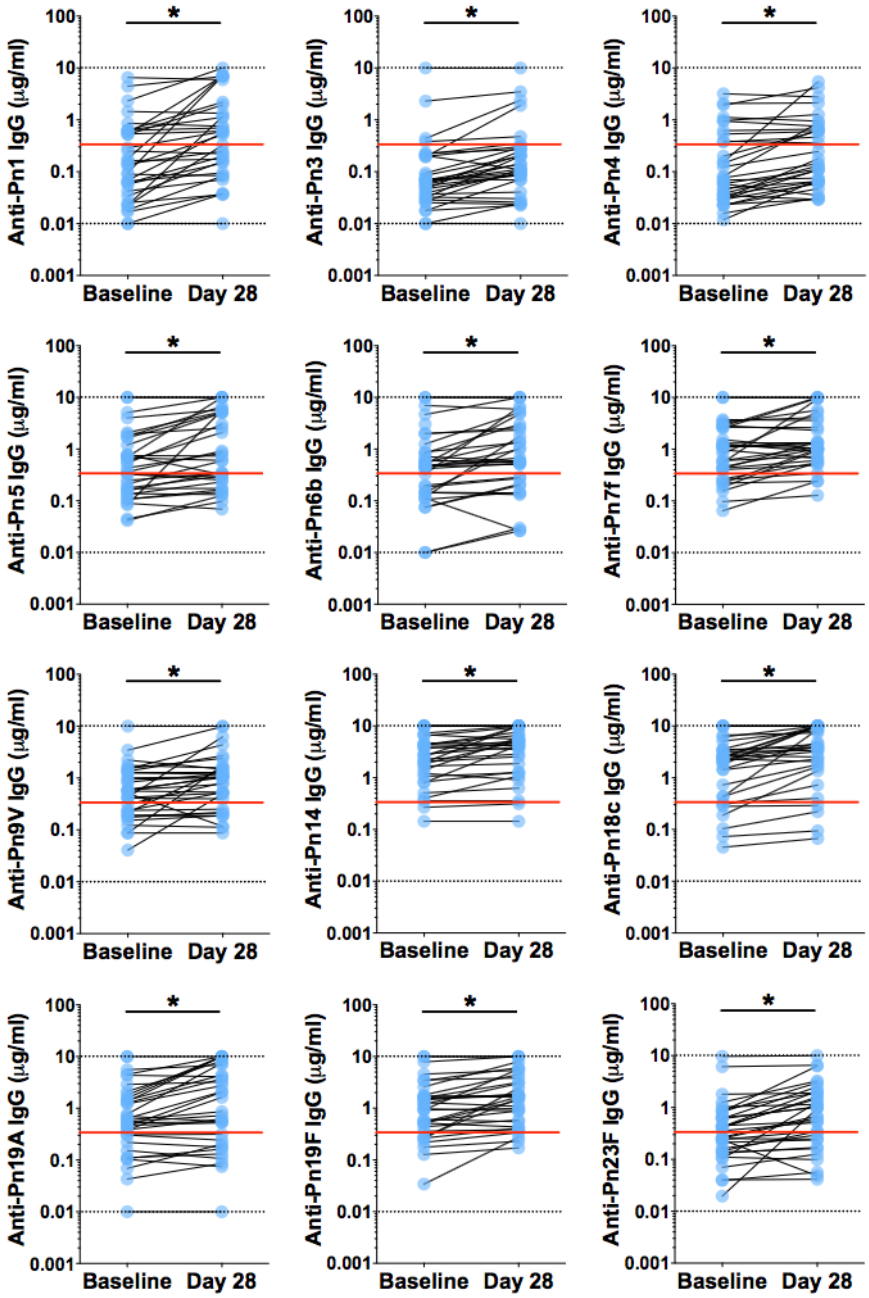
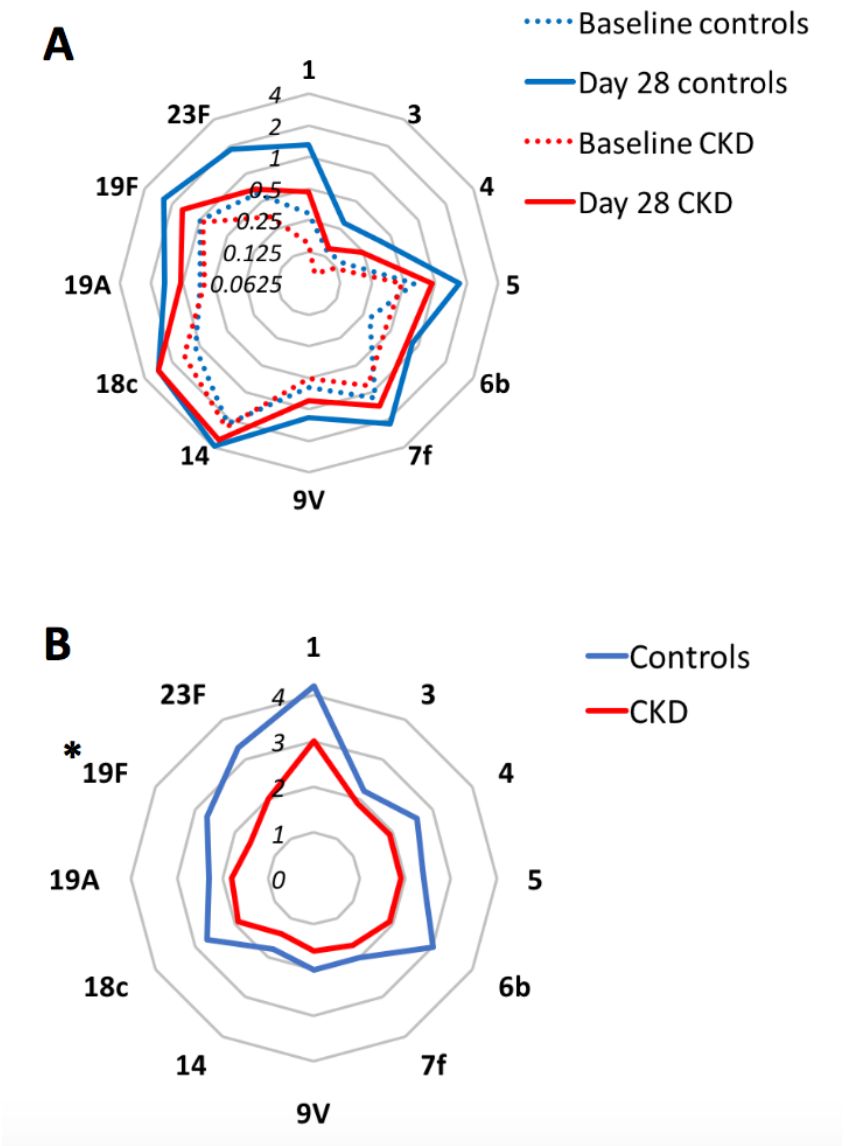


Figure 4-12 Summary of PPV23 vaccine responses in SONIC study participants.

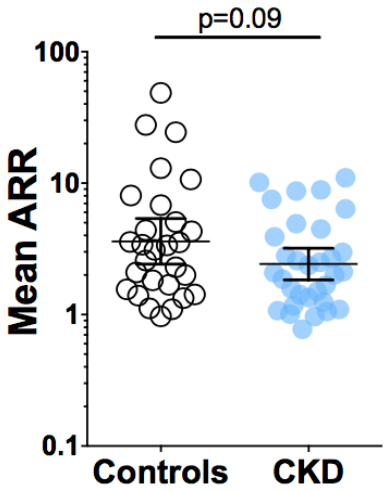
Spider diagrams of A: anti-PnPS IgG geometric mean concentrations for controls (blue) and patients with CKD (red) at baseline (dashed lines) and day 28 (solid lines) post-vaccination with PPV23; B: serotype-specific geometric mean ARR for controls (blue) and patients with CKD (red). *denotes significant difference in ARR between patient groups ($p<0.05$).



The magnitude of humoral response to individual serotypes, as measured by the antibody response ratio (ARR: geometric mean of Day 28 titre/Baseline titre, summarised in Table 4-11), was generally lower in patients with CKD than controls for all serotypes, reaching statistical significance only for serotype 19F (summarised in Figure 4-12 B). When the response to the whole vaccine was considered (calculated as the mean ARR of all 12 serotypes tested), patients with CKD tended to have a lower PPV23 mean ARR than controls, which approached statistical significance (Table 4-11 and Figure 4-13).

Figure 4-13 PPV23 whole vaccine (mean of 12 serotypes) ARR – comparison between patients with CKD and controls.

Error bars denote geometric mean and 95% CI. Unpaired t test (on log transformed data) 2-tailed p value shown.

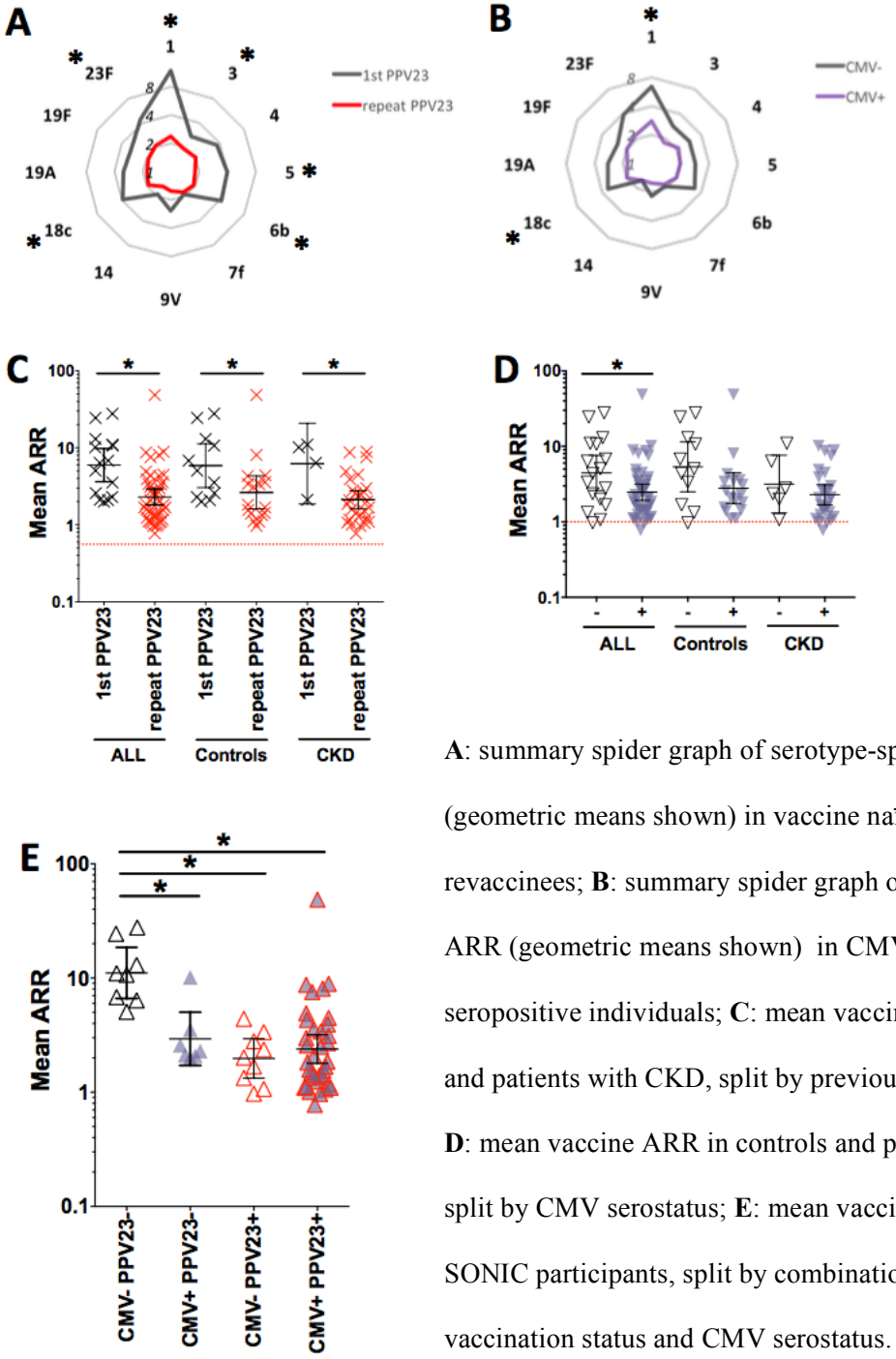


4.10 Demographic and clinical predictors of day 28 PPV23 vaccine response

When the SONIC study population was considered as a whole, a significant negative correlation was found between baseline anti-PnPS IgG concentrations and the magnitude of response (ARR) for 10 of 12 serotypes tested (1, 4, 6b, 7f, 9V, 14, 18c, 19A, 19F and 23F; Pearson's 2-tailed $p<0.05$ on log-transformed data). Age also negatively correlated with ARR for 7 of 12 serotypes tested (1, 4, 5, 6b, 9V, 14, and 19A) and mean ARR (Pearson's 2-tailed $p<0.05$ on log-transformed ratios). Few significant relationships were seen between serotype-specific ARR and measures of renal disease (eGFR – positive correlation with 19F and 23F ARR only, Pearson $p<0.05$; ACR – not significant) and multimorbidity (CCI – negative correlation with ARR for serotypes 1 and 19F, Spearman $p<0.05$; medication burden – not significant). No significant relationships were seen between ARR and markers of inflammation (NLR and hsCRP) or glycaemic control (HbA1c).

Individuals that had previously received PPV23 were significantly older than vaccine-naïve individuals (Figure 4-9). Previous vaccination with PPV23 was generally associated with lower ARR for the vaccine as a whole and for most individual serotypes, reaching significance for half of all 12 tested - 1, 4, 5, 6b, 18c and 23F (summarised in Figure 4-14, panels A and C).

Figure 4-14 Impact of previous PPV23 vaccination and latent CMV infection on magnitude of humoral response to PPV23 vaccination in SONIC study participants.



A: summary spider graph of serotype-specific ARR (geometric means shown) in vaccine naïve individuals and revaccinees; **B**: summary spider graph of serotype-specific ARR (geometric means shown) in CMV seronegative and seropositive individuals; **C**: mean vaccine ARR in controls and patients with CKD, split by previous vaccination status; **D**: mean vaccine ARR in controls and patients with CKD, split by CMV serostatus; **E**: mean vaccine ARR for all SONIC participants, split by combination of previous vaccination status and CMV serostatus. Error bars denote geometric mean and 95% CI. *denotes $p<0.05$ – unpaired t-test on log-transformed data

Interestingly, lower serotype-specific ARRs were generally seen in CMV seropositive compared to CMV negative study participants (summarised in Figure 4-14, panels B and D). Both CMV seropositivity and previous PPV23 vaccination were associated with lower mean ARR (whole vaccine response), but these variables did not have appear to have an additive effect (Figure 4-14, panel E). A significant interaction was present between these variables (univariate ANOVA p=0.004) - that is to say the impact of latent CMV infection in this study appeared to be different depending on whether or not the individual had previously been vaccinated with PPV23.

In light of the above relationships within the data, several potential confounders are present in this study of PPV23 vaccine response in controls and patients with CKD: age, baseline anti-PnPS IgG concentration, CMV serostatus and previous PPV23 vaccination status. To examine the effect of these variables on the magnitude of the humoral response to PPV23 vaccination, I performed multivariate analysis using a linear regression model that contained age, gender, baseline serotype-specific IgG concentration (for individual serotypes only), CKD status, CMV serostatus and previous PPV23 vaccination status, together with their interaction term (both positive). Previous PPV23 vaccination and CMV seropositivity, either alone or in combination ($p<0.001$ for PPV23 status, $p=0.003$ for CMV status, $p=0.004$ for interaction term), were significant predictors of lower whole vaccine (mean) ARR, independent of age, gender or CKD status (adjusted R^2 of model=0.31). This was not altered with substitution of CKD status by CCI or medication burden (as more comprehensive measures of multimorbidity). Interestingly, CMV seropositivity or previous PPV23 vaccination alone had a greater impact on mean ARR than if they were combined (interaction term): standardised Beta coefficients in the model were -0.62, -0.72 and -0.54 (adjusted), respectively. Neither CMV-specific IgG titre or

duration from last PPV23 vaccination were significant predictors of mean ARR in CMV-seropositive individuals and PPV23 revaccinees, respectively.

When this linear regression model (with addition of baseline anti-PnPS concentration) was applied to individual serotypes (Table 4-12), the most consistent significant predictor of ARR was the baseline serotype-specific IgG concentration, with CMV/PPV23 status reaching significance for only 2 serotypes (1, 18c), but approaching significance in another 4 (5, 6b, 9V and 23F). Age was also a significant predictor of ARR for serotypes 4, 6b, 9V and 14. Interestingly, CKD status reached significance as a predictor for ARR of serotype 19F, but this is likely to be a feature of multimorbidity as CCI (collinear with CKD status) was also significant (p=0.03) when substituted, in turn, for CKD status and the model fit was unchanged with subsequent addition of CKD status.

Table 4-12 Linear regression modelling of predictors of Pn serotype-specific GMRR.

P values shown for predictor variables (top row) included in linear regression model of serotype-specific GMRR (first column). Significant results (p<0.05) highlighted in bold.

	Age	Gender	Baseline serotype-specific IgG titre	CKD status	CMV status	Previous PPV23	CMV*PPV23
Pn1	0.17	0.71	**0.007**	0.66	**0.02**	**0.0001**	**0.02**
Pn3	0.74	0.79	0.28	0.65	0.10	0.28	0.40
Pn4	**0.04**	0.11	**0.002**	0.17	0.77	0.82	0.79
Pn5	0.05	0.69	0.06	0.55	0.13	0.06	0.09
Pn6b	**0.02**	0.44	**0.03**	0.36	0.11	0.17	0.08
Pn7f	0.20	0.52	**0.001**	0.13	1.0	0.55	0.79
Pn9V	**0.04**	0.29	**0.007**	0.38	0.18	0.14	0.07
Pn14	**0.04**	0.19	**<0.0001**	0.15	0.85	0.48	0.40
Pn18c	0.95	0.31	**0.01**	0.41	**0.02**	**0.02**	**0.04**
Pn19A	0.28	0.17	**0.01**	0.53	0.16	0.22	0.29
Pn19F	0.47	0.24	**0.0003**	**0.03**	0.28	0.20	0.19
Pn23F	0.95	0.24	**0.006**	0.05	**0.04**	0.08	0.08

In summary, PPV23 vaccination significantly increased serotype-specific IgG concentrations at day 28 in both patients with CKD and controls, but no independent effect of renal impairment on PPV23 humoral responses was seen in this study. Higher pre-vaccination anti-PnPS concentrations were associated with a significantly lower magnitude of vaccine-induced humoral response for most serotypes tested. Older age was also associated with poorer responses to serotypes 4, 9V and 14. Unexpectedly, both latent CMV infection and previous vaccination with PPV23, even if over a decade ago, were associated with poorer vaccine-induced responses, particularly to serotypes 1 and 18c. The association of previous PPV23 vaccination with lower serotype-specific ARR (in the face of comparable pre-vaccination serotype-specific IgG titres) is consistent with immune hyporesponsiveness to PPV23. It is surprising that this was observed even after 10 years had elapsed from previous PPV23 vaccination. As such, this warrants further investigation and consideration of the impact of vaccine efficacy. The potential impact of latent CMV infection on TI vaccines such as PPV23 also warrants further investigation in a larger cohort of older adults with and without chronic disease.

4.11 Maintenance of PPV23 response

Serotype-specific anti-PnPS IgG significantly reduced from levels achieved at day 28 post-vaccination to month 6 in patients with CKD for 9 of 12 (75%) serotypes tested (1, 3, 4, 5, 6b, 7f, 19A, 19F and 23F), but only 5 of 12 (42%) in controls (1, 4, 6b, 7f and 23F) as summarised in Table 4-11, Figure 4-15, Figure 4-16 and Figure 4-17.

Figure 4-15 Serotype-specific anti-PnPS IgG at day 28 and month 6 post-vaccination in individual controls.

Range of assay: 0.01 -10 microgram/m (dashed lines): all values below 0.01 were assigned a value of 0.01, all values above 10 were assigned a value of 10. Red line denotes WHO long-term protective threshold of 0.35microgram/ml. *denotes $p<0.05$ paired t-test performed on log-transformed data.

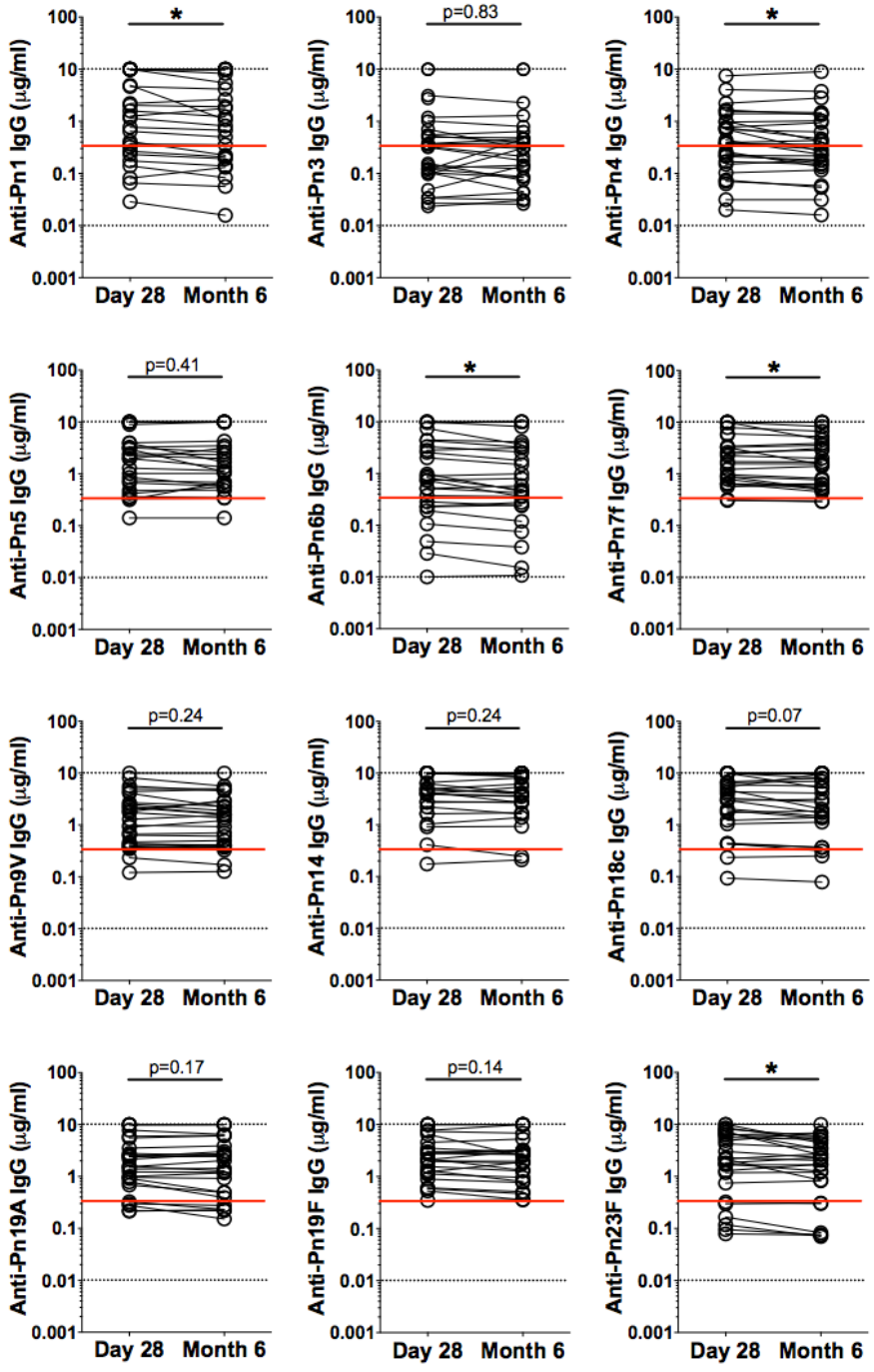


Figure 4-16 Serotype-specific anti-PnPS IgG at day 28 and month 6 post-vaccination in individual patients with CKD.

Error bars denote geometric mean and 95% CI. Range of assay: 0.01 -10 microgram/m (dashed lines): all values below 0.01 were assigned a value of 0.01, all values above 10 were assigned a value of 10. Red line denotes WHO long-term protective threshold of 0.35microgram/ml. *denotes $p<0.05$ paired t-test performed on log-transformed data.

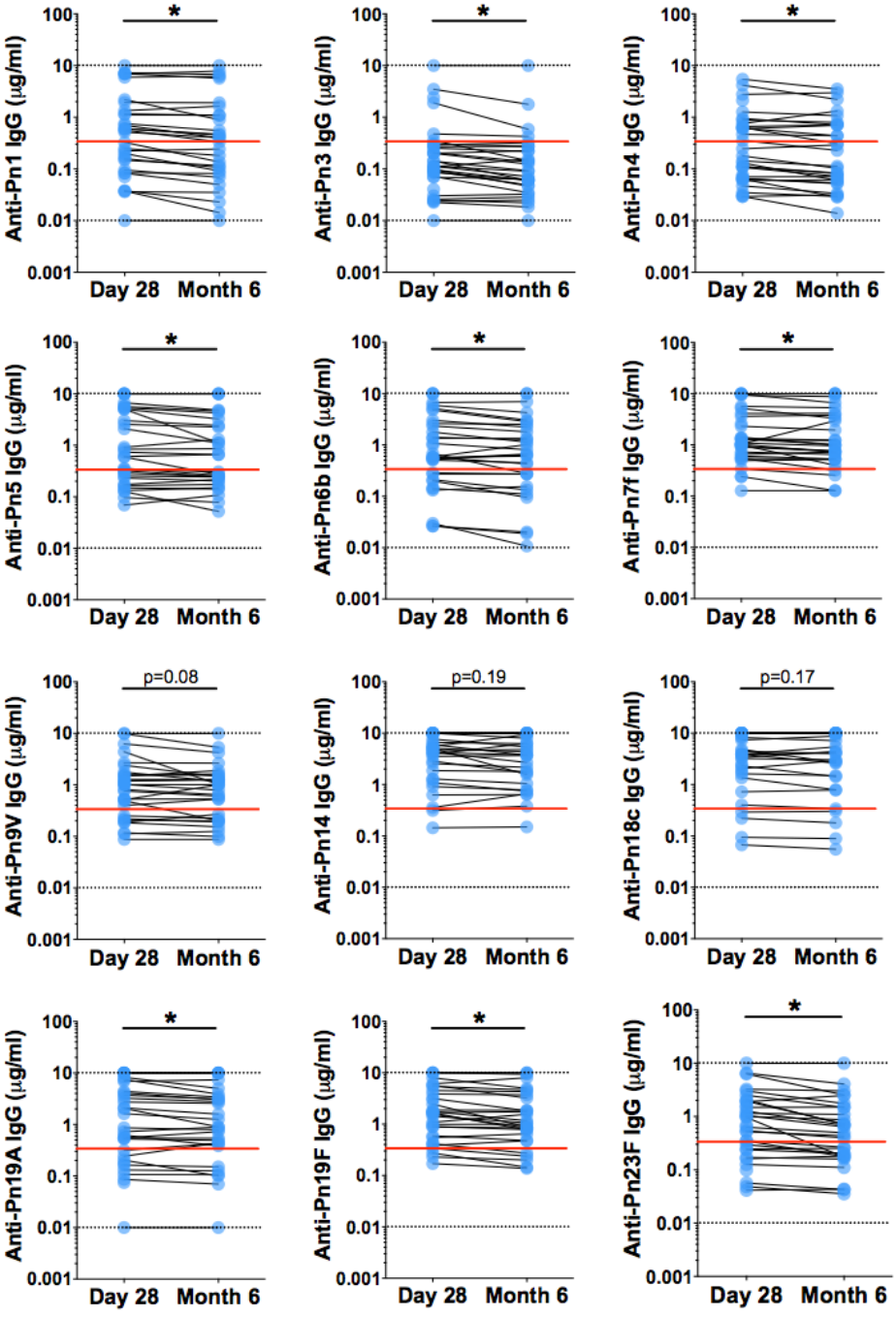
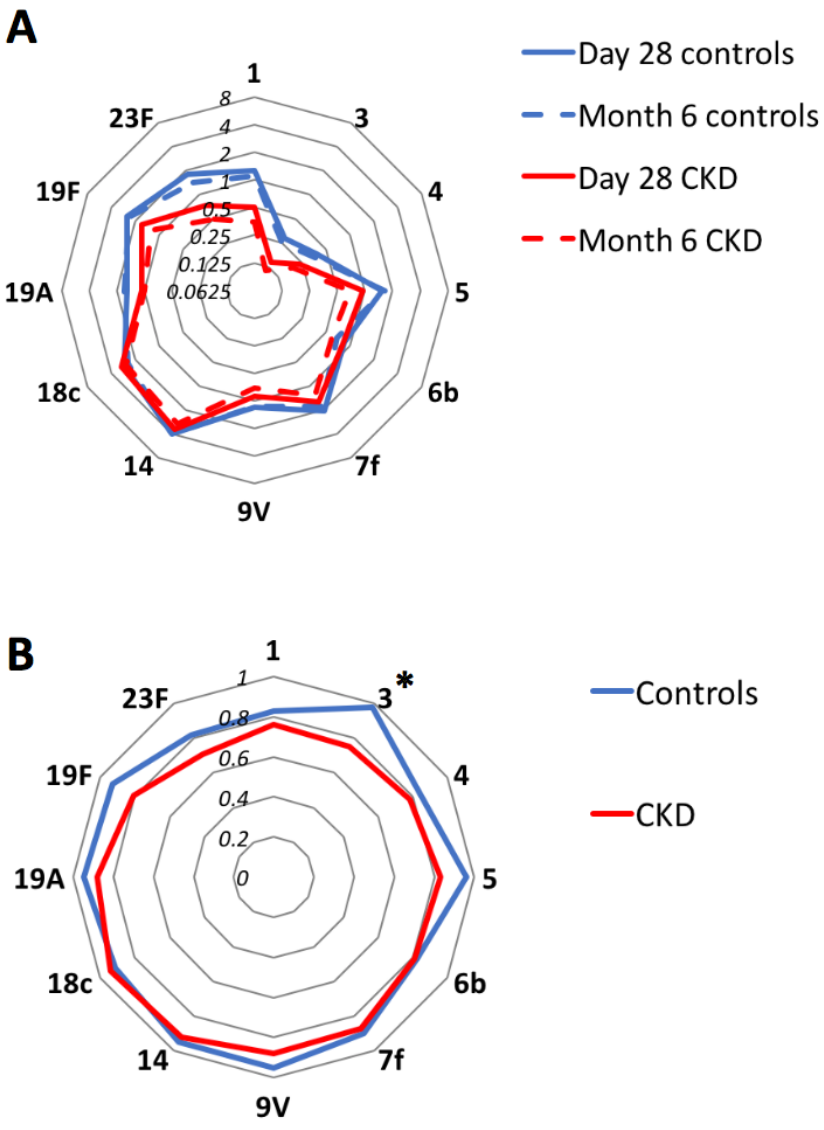


Figure 4-17 Summary of maintenance of PPV23 response in SONIC study participants.

Spider diagrams of A: anti-PnPS IgG geometric mean titres for controls (blue) and patients with CKD (red) at day 28 (solid lines) and month 6 (dashed lines) post-vaccination with PPV23; B: serotype-specific geometric mean AMR for controls (blue) and patients with CKD (red). *denotes significant difference in AMR between patient groups ($p<0.05$).



The magnitude of PnPS-specific IgG change at month 6 from peak titres at day 28 was assessed using the antibody maintenance ratio (AMR: geometric mean of month 6 titre/day 28 titre), where a value of 1 represents maintenance of titre and values less than 1 represent a reduction. Serotype-specific AMR was generally lower in patients with CKD than controls for the majority of serotypes tested, reaching significance for serotype 3 (Table 4-11, summarised in Figure 4-17). This mirrors the pattern seen at baseline where anti-Pn3 IgG was slightly lower in CKD revaccinees than controls.

When maintenance of response to PPV23 was considered as a whole (using the mean AMR for all 12 serotypes tested), there was a downward trend in patients with CKD that approached statistical significance (Table 4-11). Interestingly, mean AMR was significantly lower than 1 in patients with CKD (one-sample unpaired t-test on log transformed data, 2-tailed p=0.001) but not in controls, suggesting that, overall, patients with CKD did not maintain peak anti-PnPS IgG levels to the vaccine as a whole at month 6. This is similar to the findings described earlier with responses to TIV (summarised in Figure 4-6).

Both patient groups had significantly higher anti-PnPS IgG concentrations at month 6 than pre-vaccination for all serotypes tested (Table 4-11), but revaccinees, per individual, had significantly fewer serotypes for which month 6 titres exceeded baseline (Mann Whitney 2-tailed p=0.04). Indeed, month 6 titres were greater than baseline in at least 8 of 12 serotypes tested for all 1st time PPV23 recipients, whereas this was not the case in 17 revaccinees (40%, Fisher's exact p=0.005).

4.12 Clinical predictors of PPV23 maintenance

Serotype-specific ARR (peak response ratio) negatively correlated with AMR (maintenance ratio) for 11 of 12 serotypes tested (1, 3, 4, 5, 6b, 7f, 9V, 14, 19A, 19F and 23F; Pearson's 2-tailed $p<0.05$ on log-transformed data). As seen with responses to TIV, this may simply indicate that a robust response to PnPS is short-lived, with greater "maintenance" of titre reflecting little change in anti-PnPS IgG across the study for individuals with poor PPV23 responses. Age, multimorbidity as measured using CCI, glycaemic control (HbA1c), CMV-specific IgG titre and hsCRP were not significantly associated with serotype-specific or mean AMR in this study. Unlike with ARR, no significant differences in AMR were seen between PPV23 naïve individuals and revaccinees, or between CMV seronegative and seropositive individuals in this study.

In keeping with the above relationships within the data, serotype specific ARR was a significant predictor of AMR for all serotypes except 18c in a linear regression model that also included age, gender and CKD status. CKD status was also a significant predictor of AMR for serotypes 3 and 19F in this model. However, when CKD status was substituted with its collinear variable CCI (a more comprehensive measure of multimorbidity), the new variable was also significant. No improvement in model fit was seen with subsequent addition of CKD status, suggesting that multimorbidity appears to explain the variability in AMR for serotypes 3 and 19F better than the presence or absence of renal impairment in this study. However, patients with CKD in this study were significantly more comorbid than controls, so a significant impact of CKD here cannot be ruled out.

Overall, these data suggest that robust peak responses to PPV23 vaccination are not maintained at 6 months in older adults with and without chronic disease. No independent

effect of renal impairment was seen in this study, but reduction from peak for anti-PnPS IgG against serotypes 3 and 19 was greater in more comorbid individuals (who frequently had concomitant CKD). Overall, anti-PnPS IgG concentrations remained above baseline levels for all serotypes tested in both patient groups. However, significantly fewer revaccinees had month 6 anti-PnPS concentrations that exceeded pre-vaccination levels for 8 of 12 serotypes tested, compared to 1st time PPV23 recipients. This, together with reduced magnitude of peak response to PPV23, supports the hypothesis of PPV23-induced hyporesponsiveness.

4.13 Clinical parameters of adequate PPV23 vaccine response

There are two main clinical criteria to assess adequacy of the immune response to PPV23 (Table 4-13).

The AAAAI criteria were designed as a diagnostic tool for identifying/assessing immunodeficiency states (287) and are therefore more stringent than the WHO criteria (31), which were developed to assess antigen immunogenicity in vaccine development. Both criteria can be applied to 1st time vaccine recipients and revaccinees. AAAAI criteria were developed for use in children and adults under the age of 65, so may over-estimate immunodeficiency in the elderly.

A serotype-specific anti-PnPS IgG titre of 0.35μg/ml is associated with long-term protection against infection with that specific pneumococcal serotype (31) and will henceforth be referred to as the "WHO protective threshold".

Table 4-13 Clinical criteria to assess adequacy of PPV23 response.

AAAAI: American Academy of Asthma, Allergy and Immunodeficiency; WHO: World Health Organisation. Table compiled from references (31, 54).

	AAAAI	WHO
Threshold titre	1.3µg/ml	0.35µg/ml
Adequate response criteria	conversion of titre below threshold to above OR 2-fold increase if pre-vaccination titre above threshold for >70% serotypes tested (8/12)	

At baseline, there were no significant differences between controls and patients with CKD in the number of individuals with anti-PnPS IgG concentrations above the protective threshold for any of the 12 serotypes tested (summarised in Table 4-14). No significant differences were seen when the study group was also split by previous vaccination status. Serotypes with the lowest proportion of study participants having anti-PnPS IgG concentration above the WHO protective threshold were 1, 3 and 4.

The proportion of individuals with anti-PnPS IgG at or above the WHO protective threshold generally increased following vaccination in both controls and patients with CKD. However, the proportion of individuals with protective levels to 8 or more serotypes significantly increased at day 28 post-vaccination from baseline only in controls (Fisher's exact p=0.002) and not in patients with CKD (Fisher's exact p=0.32) - Table 4-14.

Using WHO criteria for adequate response, significantly fewer patients with CKD had adequate responses to serotypes 1 and 3, with trends for reduced responses to serotypes 5 and 19F (Table 4-13). Patients with CKD also had a significantly lower number of

serotypes for which an adequate response was achieved per person, compared to controls (median 3 (IQR 4) versus 5 (6), respectively, Mann-Whitney 2-tailed p=0.03). When response to PPV23 as a whole was considered, approximately 1 in 5 individuals (7 controls (24%) and 6 patients with CKD (17%), Fisher's exact 2-tailed p=0.54) had an adequate response to 8 of 12 serotypes tested.

Table 4-14 WHO PPV23 vaccine response adequacy criteria – number of individuals with adequate response by serotype.

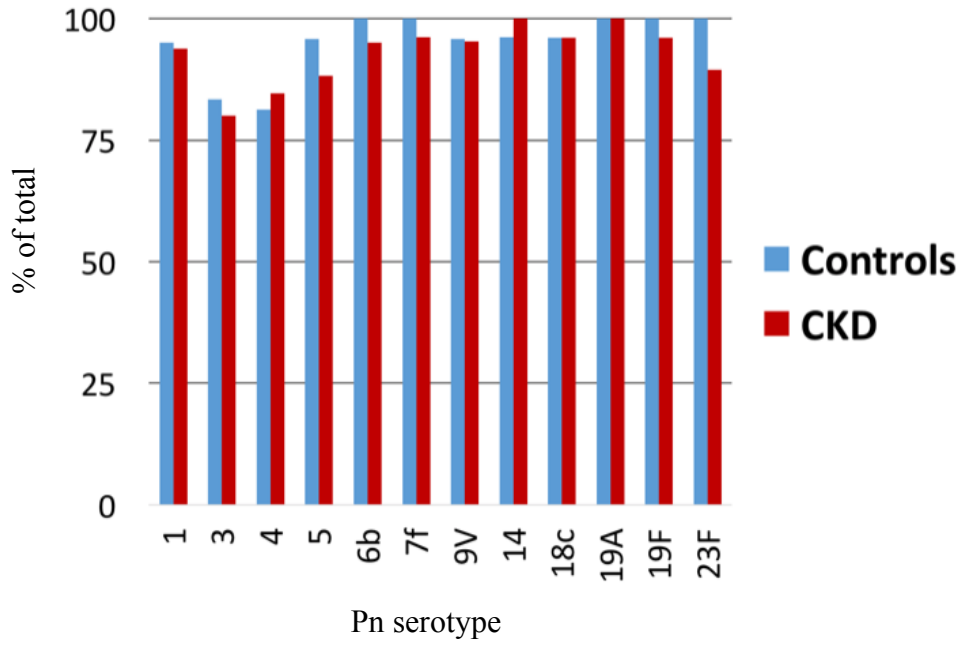
N and (%) shown unless stated. Statistical comparisons performed between controls and patients with CKD. *denotes Fisher's exact 2-tailed p<0.05, ~ denotes Mann-Whitney 2-tailed p<0.05.

	Controls			CKD		
	n=29 ≥WHO at baseline	n=28 ≥WHO at day 28	Adequate response	n=33 ≥WHO at baseline	n=32 ≥WHO at day 28	Adequate response
Pn1	12 (41)	20 (71)	17 (61)	10 (30)	17 (53)	10 (31)*
Pn3	5 (17)	13 (46)	10 (36)	4 (12)	6 (19)	3 (9)*
Pn4	8 (28)	16 (57)	10 (36)	10 (30)	14 (44)	6 (19)
Pn5	17 (59)	24 (86)	17 (61)	17 (52)	19 (59)	11 (34)
Pn6b	14 (48)	20 (71)	11 (40)	20 (61)	22 (69)	9 (28)
Pn7f	23 (79)	26 (93)	13 (46)	25 (76)	28 (88)	10 (31)
Pn9V	21 (72)	25 (89)	11 (40)	20 (61)	24 (75)	8 (25)
Pn14	27 (93)	27 (96)	8 (29)	31 (94)	30 (94)	6 (19)
Pn18c	24 (83)	26 (93)	14 (50)	26 (79)	28 (88)	12 (38)
Pn19A	20 (69)	22 (79)	9 (32)	23 (70)	22 (69)	12 (38)
Pn19F	23 (79)	27 (96)	14 (50)	26 (79)	28 (88)	9 (28)
Pn23F	19 (66)	22 (79)	13 (46)	17 (52)	21 (66)	13 (41)
No. serotypes per person: median (IQR)	7 (5)	10 (3)	5 (6)	7 (4)	9 (4)~	3 (4)~
≥8/12 serotypes	13 (45)	24 (86)	7 (25)	16 (48)	20 (63)	6 (19)

Over 80% of controls and patients with CKD that had reached the WHO protective threshold at day 28 post-vaccination maintained protective levels of anti-PnPS IgG at month 6, without a significant difference seen between the groups (Figure 4-18). All but one individual (a control subject) that had protective levels of anti-PnPS to 8 or more of the serotypes tested maintained protective levels to 8 or more serotypes at month 6.

Figure 4-18 Proportion of individuals that maintained anti-PnPS IgG concentrations at or above the WHO protective threshold at month 6.

Blue – controls, red – patients with CKD.



Using AAAAI PPV23-deficiency phenotype definitions, both controls and patients with CKD showed surprisingly poor humoral immune responses (Table 4-15) with 24 controls (86%) and all but 1 patient with CKD meeting criteria for moderate/severe deficiency phenotypes.

Table 4-15 AAAAI PPV23 deficiency phenotypes – comparison between controls and patients with CKD.

This analysis does not account for infection status and baseline total immunoglobulins/subclasses.

N (%) and Fisher's exact 2-tailed p shown. P<0.05 considered significant.

PPV23 deficiency phenotype	Controls n=28	CKD n=32	p value
None	4 (14)	1 (3)	0.17
Mild Anti-PnPS IgG titre ≤1.3µg/ml to >1 serotype **OR** 2-fold increase for <70% serotypes tested if prevaccination titre >1.3 but ≤4 µg/ml	0 (0)	0 (0)	1.0
Moderate Anti-PnPS IgG titre ≥1.3µg/ml to <70% serotypes	13 (47)	9 (28)	0.18
Severe Anti-PnPS IgG titre ≥1.3µg/ml to 2 or fewer serotypes	11 (39)	21 (66)	0.07

For diagnosis of specific antibody deficiency (one of the main primary immunodeficiency syndromes associated with isolated poor anti-polysaccharide responses), AAAAI vaccine criteria are combined with a history of recurrent respiratory infections and an otherwise normal adaptive immune system. Taking into account baseline immunoglobulin and IgG subclass levels, together with history of recurrent (2 or more) respiratory infections in the 6 months following vaccination, one patient with CKD (but no controls) did fulfil AAAAI

humoral criteria for specific antibody deficiency, although this definition was developed for adults aged less than 65 years.

PPV23 “responder” status was associated with significantly younger age compared with “non-responder” status for both WHO (median 67 years (IQR 9.5) in responders, 75 (11) in non-responders, Mann Whitney 2-tailed p=0.002) and AAAAI criteria (67 (9.5) in responders and 75 (11) in non-responders, p=0.03). AAAAI, but not WHO responders had lower CCI (median score=0 (IQR 3) versus 3 (4), Mann Whitney 2-tailed p=0.08) and higher eGFR (median 90 (IQR 42) versus 38 (53), Mann Whitney 2-tailed p=0.03) than non-responders in this study. However, no significant differences were seen between PPV23 responders and non-responders (either WHO or AAAAI definition) in proteinuria (ACR), inflammation (hsCRP) and glycaemic control (HbA1c). No significant differences in proportions of individuals seropositive for CMV were seen between responders and non-responders, as defined by either clinical criteria.

Although both clinical adequacy criteria are designed to be used to assess both 1st and repeat vaccination responses, a greater proportion of non-responders had previously received PPV23 than responders (WHO criteria: 39/47 versus 6/13, Fisher’s exact 2-tailed p=0.01; AAAAI criteria: 43/55 versus 2/5, p=0.09). However, previous PPV23 recipients were significantly older than vaccine-naïve individuals (Figure 4-9), so this may simply reflect the difference in age between responders and non-responders. The absolute number of PPV23 responder individuals in this study, using either clinical criteria for adequate response, is too small for meaningful multivariate analysis using logistic regression. As such, it is not possible to tease out the independent effects of age and previous PPV23 vaccination in this analysis.

In summary, using clinical criteria to assess vaccine response adequacy, the immune response to PPV23 is very poor in older adults with and without chronic disease with approximately only 1 in 5 fulfilling WHO criteria for adequate response to the whole vaccine and over 85% potentially fulfilling AAAAI criteria for humoral immunodeficiency. There is also a subtle reduction in the adequacy of immune response as defined by WHO criteria in patients with CKD compared to controls, with greatest divergence between the groups for serotypes 1 and 3. These two serotypes are associated with invasive pneumococcal disease, which confers a high mortality rate in older adults. At baseline, these two invasive serotypes had the lowest proportion of individuals with WHO protective titres. As such, even a subtle reduction in vaccine response adequacy to these serotypes, as seen in patients with CKD in this study, may have significant clinical consequences. Advancing age and previous PPV23 were associated with PPV23 non-responder status, as defined by either clinical criteria, but the independent impact of these variables is unclear.

4.14 Relationship of TIV/PPV23 vaccine response to incidence of infection

No significant differences were seen in baseline titres against any of the 3 flu strains contained in TIV or any of the 12 pneumococcal serotypes tested between individuals that did or did not report infections in the 6 months of study follow-up. Pneumococcal strain-specific ARR or AMR were also equivalent between those that did or did not report infections, but ARR for influenza strain A/H3N2 was significantly higher in controls that reported infections than controls that did not (median 1.0 (IQR 3) and 2.7 (4),

respectively; unpaired t-test on log-transformed data 2-tailed p=0.04). However, this significance was lost when only respiratory infections were considered. No significant differences were seen in proportion of responders and non-responders for TIV or PPV23 reporting infections in the 6 months of study follow-up.

4.15 Discussion

This Chapter describes the humoral response to both a T-dependent (TIV) and T-independent vaccine (PPV23) in older adults with and without moderate/severe CKD. Although both controls and patients with CKD were able to increase strain-specific influenza HAI titres and anti-PnPS IgG at day 28 post-vaccination and largely to a similar degree, approximately 1 in 5 older adults with and without CKD fulfilled clinical criteria for adequacy of vaccine response for either TIV or PPV23. These poor responses in older adults with and without chronic disease are largely in keeping with previous observations of declining vaccine responses with advancing age (64, 275, 280). Indeed, age was one of the main predictors of poor vaccine responses, together with higher baseline antigen-specific HAI or IgG titres (also previously reported for TIV (288) and PPV23 (55)). Multimorbidity and renal impairment were both generally associated with poorer vaccine responses, but an independent effect of renal impairment could not be conclusively elucidated.

Multiple comparisons have been made between data from patients with CKD and controls in this chapter, without a formal Bonferroni correction. Although some of the significant findings may represent type I errors (false positives), most are probably due to genuine differences between the disease groups and associations between variables.

A history of previous PPV23 vaccination (even if over a decade ago) was associated with reduced humoral responses to repeat PPV23 in both controls and patients with CKD. This is consistent with immunological hyporesponsiveness to PPV23, which has previously been variably reported in older adults (55, 56, 58, 60, 66, 289). Several potential reasons for this variability exist, including differences in the definition of hyporesponsiveness and interpretation of its clinical significance between studies, together with differences in the number and combination of pneumococcal serotypes against which antibody responses are tested. For example, although in the study by Kawakami et al (55) a reduction in magnitude of response following repeat PPV23 to all 14 serotypes tested was noted in older adults, the authors conclude that this is due to higher pre-vaccination titres in revaccinees without multivariate analysis to examine the independent effect of previous PPV23 vaccination.

As described in Chapter 1 (Introduction), one hypothesis for hyporesponsiveness in response to repeated exposures to plain polysaccharide vaccines is the depletion of antigen-specific memory B cells. Activation of these memory B cells may drive their differentiation into ASCs, without replenishing or increasing the size of the memory B cell population. This concept is supported by the results of an elegant study from Clutterbuck et al (25), where PPV23 vaccination induced a reduction in circulating memory B cell populations and also impaired subsequent memory B cell expansions in response to a 7-valent pneumococcal conjugate vaccine (PCV-7). Although, anti-PnPS IgG concentrations generally remained above baseline levels for all serotypes tested in both patient groups in the SONIC study, month 6 titres exceeded baseline levels for at least 8 of 12 serotypes in all 1st time PPV23 recipients, whereas this was not the case in 40% of revaccinees. This is

consistent with a depletion of B cell antigen-specific memory in response to repeat exposure to plain polysaccharide antigens.

There a number of weaknesses of the analysis of humoral PPV23 response in this study. Firstly, I did not characterise antigen-specific IgM, IgA or subclasses of IgG induced by vaccination in patients with CKD compared to controls, which may have yielded further insights into both the nature of the immune response to PPV23 in older adults and the differences between disease groups. I also did not evaluate the function of the antigen-specific IgG produced e.g. using an opsono-phagocytic assay such as described in (290). However, the findings presented in this study, particularly the presence of hyporesponsiveness to pneumococcal polysaccharides even if previous exposure was over a decade ago, in addition to previous studies demonstrating diminished responses to PPV23 in the elderly, suggest that we may need to re-evaluate the role and effectiveness of PPV23 in this vulnerable patient group in the post-PCV13 era.

An unexpected finding in this study is the independent effect of latent CMV infection on humoral responses to PPV23, with CMV-seropositive individuals demonstrating generally reduced responses. Although CMV-associated impairment in B cell responses has previously been reported with regard to T-dependent vaccines (96), this has not previously, to my knowledge, been shown for T-independent antigens. As such, this requires confirmation in a larger cohort of CMV seropositive and seronegative older adults.

In this study, comprehensive interrogation of the humoral response to TIV was limited by the small sample size overall and heterogeneity of vaccine composition across the 3 years that the study was run, which further reduced sample size in “like-for-like” analysis. Even

when using clinical parameters of adequate vaccine response (thus maximising total sample size here), to observe a significant reduction in proportion of patients with CKD mounting an adequate response to TIV compared to controls of the same magnitude as demonstrated in this study (27% patients with CKD versus 43% controls) with 80% power at a confidence level of 5% would require a total sample size of approximately 280 individuals (140 in each disease group). Therefore, I may not have seen a difference between patients with CKD and controls, when it exists, due to small sample size. I also did not evaluate vaccine-induced responses to other influenza antigens e.g. neuraminidase (NA), which may have yielded further insights into differences in responses to TIV between patients with CKD and controls.

Despite the similarity of vaccine responses between the patient groups, individuals with CKD reported a significantly greater number of respiratory infections over the 6 month study follow-up period. This observation suggests that there may be other factors at play in the observed immunodeficiency state in CKD, other than Ab production. As described in Chapter 1 (Introduction), multiple defects in both innate and adaptive immune responses have been previously reported in patients with CKD/ESRD. In the next 3 Chapters of this thesis, I will present results of cross-sectional lymphocyte phenotype analysis and neutrophil function, which demonstrate defects that may be responsible for the increased susceptibility to infection observed in this study.

Another possibility for the observed lack of difference in vaccine response between the disease groups in this study, in the face of significantly greater infections reported by patients with CKD, is that the vaccines used here may not represent a good “mimic” of natural infection. The largest body of evidence regarding impaired vaccine responses in CKD/ESRD is with the hepatitis B vaccine (154, 179). The most commonly used

preparation is Engerix B (GSK, Belgium), which contains a non-infectious recombinant hepatitis B virus surface antigen (HBsAg) derived from genetically engineered *Saccharomyces cerevisiae* cells, with the vaccine containing less than 5 % yeast protein (291). This recombinant HBsAg is also adsorbed on aluminium hydroxide (alum), which acts as an adjuvant in the. Although Engerix B is a T-dependent vaccine, similar to seasonal TIV, the latter does not contain any adjuvants. As described in Chapter 1, Table 1-5, alum exerts a number of immune effects including induction of local secretion of cytokines and chemokines, monocyte/macrophage recruitment and enhanced antigen presentation (292). The method of HBsAg manufacture may also mean that other PAMPs e.g. zymosan (interacts with TLR2/6) from *Saccharomyces cerevisiae* cells are incorporated into the vaccine. As such, the mechanisms of immune responses to the two T-dependent vaccines Engerix B and seasonal TIV are likely to be different. It is possible that defects in antigen presentation, TLR signalling and innate immune system activation are more prominent in CKD-associated immune dysfunction than lymphocyte response mechanics and therefore studies using Engerix B have shown more marked divergence in responses between patients with CKD and controls. Indeed, previous studies (albeit in dialysis populations) have shown reduced circulating numbers of DCs and impaired DC function (293, 294). Responses to TIV in this study may not, therefore, represent a true mimic of responses that could be elicited during natural infection. This could explain why vaccine responses appeared to be similar between the two disease groups, but self-reported infection rates were higher in patients with CKD compared to controls.

CHAPTER 5

T LYMPHOCYTES IN OLDER ADULTS WITH CKD

5.1 Introduction

T lymphocytes play a key role not only in direct pathogen killing, but also in helping B lymphocytes generate high affinity class-switched antibody (3). As I have used two vaccines as an "in vivo" antigen challenge in this study of older individuals with CKD, I thought it pertinent to examine the composition of the $CD4^+$ T cell compartment alongside analysis of vaccine-specific IgG antibody.

As described in Chapter 1 (Introduction), several previous studies have suggested that the changes in the T lymphocyte compartment seen in patients with CKD/on dialysis may be consistent with a hypothesis of accelerated "immune ageing" (142). However, these T cell phenotypes have also previously been reported in association with latent CMV infection (86). The design of this study provided a unique opportunity to explore these age- and CMV-associated T cell populations in a well-phenotyped cohort of older adults with and without CKD.

In this Chapter I will present results of cross-sectional T lymphocyte phenotyping performed on patients with CKD and age-matched controls, either at the baseline study visit (pre-vaccination) or at month 6 after vaccination. These study timepoints were deemed suitable for cross-sectional phenotyping as they represent a "background" immune state, rather than one recently altered by the study vaccinations.

During lymphocyte analysis, 2 individuals (1 patient with CKD and 1 control subject) were identified to have abnormally large proportions of B cells (>50% total lymphocytes), but with normal total circulating lymphocyte counts. Although there were no other clinical features to suggest a haematological malignancy, such expansions are in keeping with monoclonal B-cell lymphocytosis (MBL) - a pre-malignant condition with a 1-2% annual

risk of progression to chronic lymphocytic leukaemia (CLL) (295). Genetic mutations associated with CLL have been reported in MBL (296) and this condition, therefore, represents an abnormal immune system. In view of this, I chose to exclude these 2 individuals from the final lymphocyte phenotyping analysis.

5.2 Latent CMV infection has a greater impact on $CD4^+$ and $CD8^+$ T cell proportions than CKD

Figure 5-1 shows representative gating for identification of $CD4^+$ and $CD8^+$ T cells from frozen PBMCs. T cell proportion of lymphocytes were equivalent between controls and patients with CKD (Figure 5-2). Proportions of $CD4^+$ and $CD8^+$ T cells were also equivalent between the disease groups, as was the CD4/8 ratio (less than 1 (or inverted) in 1 control and 2 patients with CKD).

Figure 5-1 Representative gating strategy for identifying CD4+ and CD8+ T cells from frozen PBMCs.

Scatter plots shown. Lymphocytes were identified based on forward scatter (FSC) and side-scatter (SSC) characteristics. Singlet lymphocytes were identified by FSC area and height and live lymphocytes were gated as APC-Cy7 negative. T cells were defined by the presence of surface CD3 and further sub-categorised based on CD4 and CD8 expression.

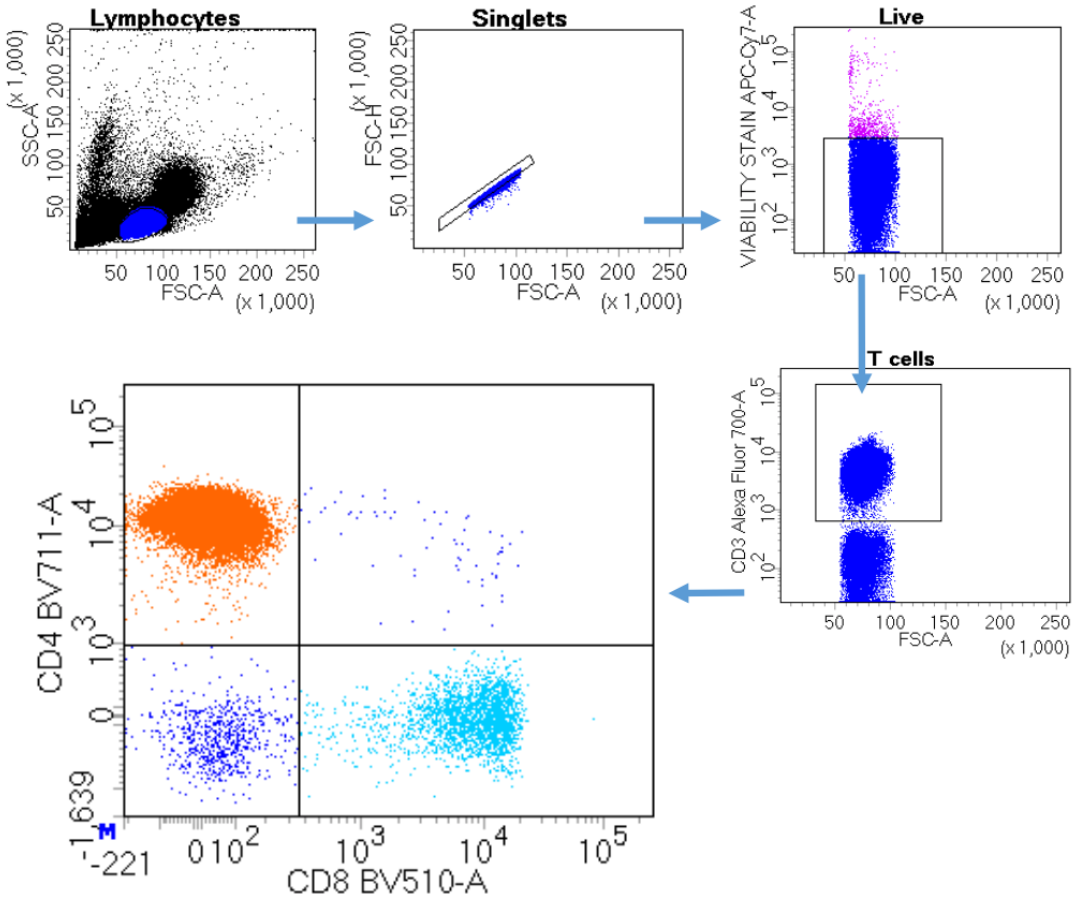
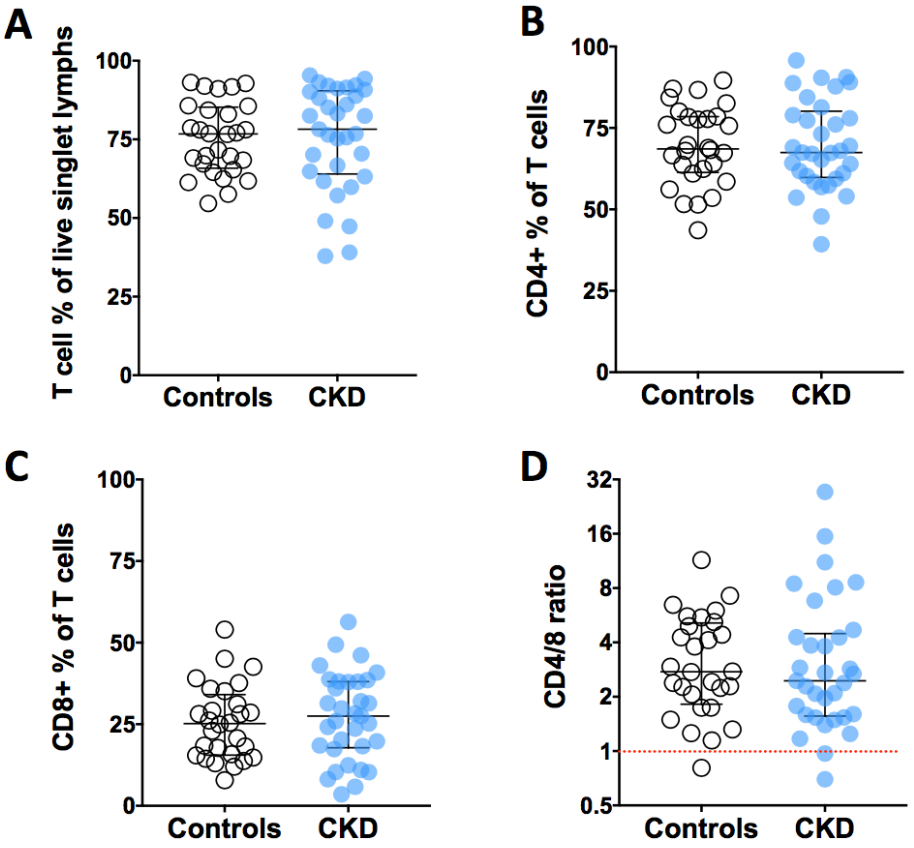


Figure 5-2 Basic T cell phenotyping – comparison between controls and patients with CKD.

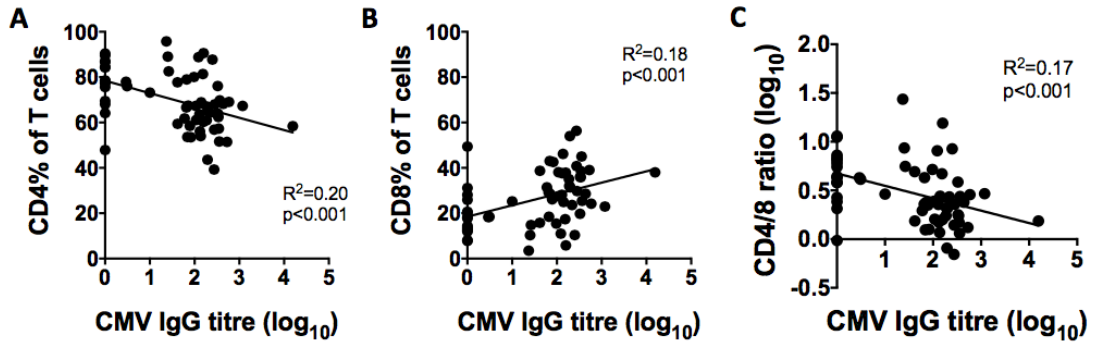
Total T cell, CD4+ and CD8+ proportions (A-C) and CD4/8 ratio (D) shown for controls (unfilled symbols) and patients with CKD (blue symbols). Error bars show median and IQR. Red dashed line in panel D denotes CD4/8 ratio of 1.



Neither total T cell proportion of lymphocytes, nor $CD4^+$ or $CD8^+$ proportion were significantly associated with measures of CKD severity (eGFR, ACR), multimorbidity (CCI, medication burden), inflammation (hsCRP) or glycaemic control (HbA1c). However, lower $CD4^+$ and higher $CD8^+$ proportions of T cells were significantly associated with higher CMV-specific IgG titre (Figure 5-3).

Figure 5-3 Relationship of CMV-specific IgG titre with basic T cell phenotype.

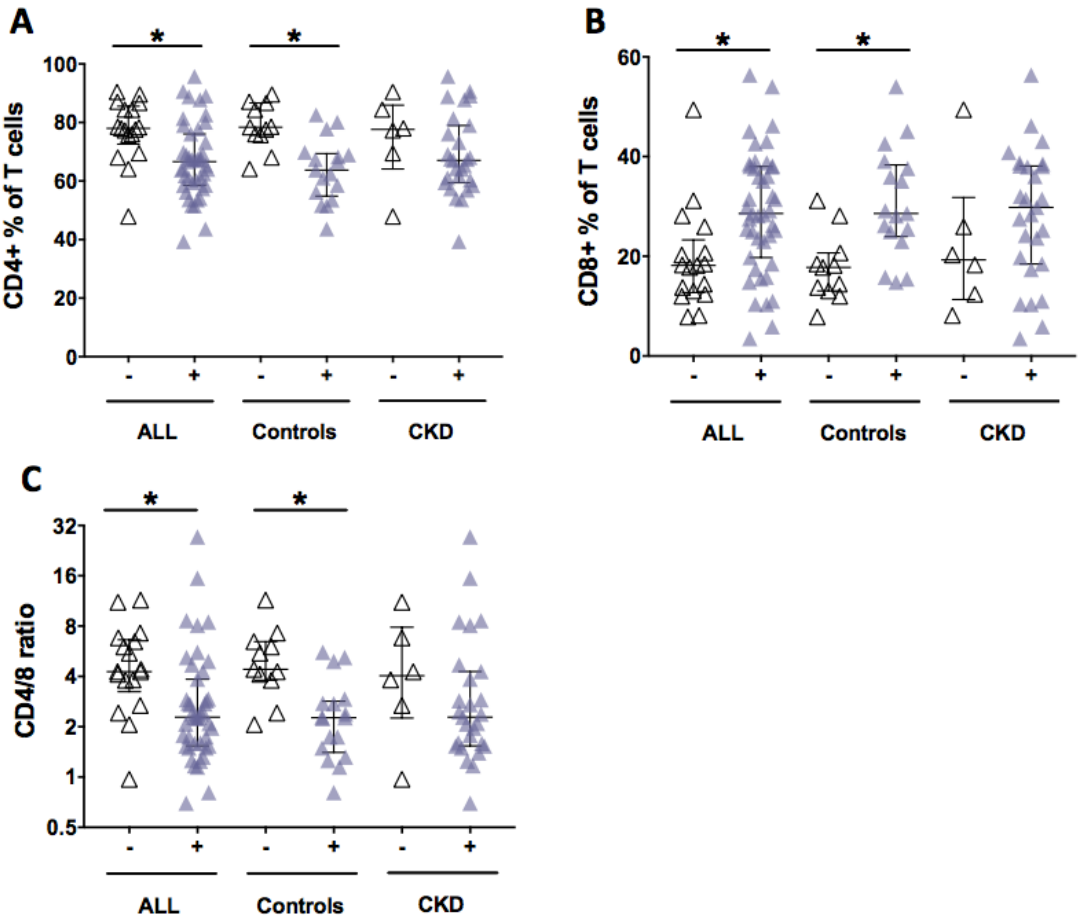
Linear regression statistics shown for relationships between CMV-specific IgG titre and proportions of CD4+ (A) and CD8+ (B) T cells, together with CD4/8 ratio. P value <0.05 considered significant.



As described in Chapter 3 (Characterisation of the study cohort), patients with CKD in this study had a slight excess of CMV-seropositive individuals (defined by a CMV-specific IgG titre ≥10 AU as previously described (180)) compared to controls (83% versus 63%, Fisher's exact 2-tailed p=0.09). However, the titre of CMV-specific IgG in seropositive individuals was equivalent between the two patient groups, suggesting a similar CMV immune "imprint" (95). When study participants were split by CMV serostatus, both CMV seropositive patients with CKD and controls generally had higher $CD8^+$ proportions T cells, mirrored by a reduction in $CD4^+$ proportions and the CD4/8 ratio (Figure 5-4). No significant differences in $CD4^+$ or $CD8^+$ proportions of T cells were seen when CMV seropositive or seronegative individuals were compared by disease group.

Figure 5-4 Effect of the presence of latent CMV infection on basic T cell phenotypes.

Comparisons between CMV seronegative and seropositive individuals in proportion of CD4+ (A) and CD8+ (B) T cell, together with CD4/8 ratio (C). Lilac colour represents data from CMV-seropositive (+) individuals, unfilled symbols represent data from CMV-seronegative (-) individuals. Error bars show median and IQR. *denotes Mann Whitney 2-tailed p<0.05

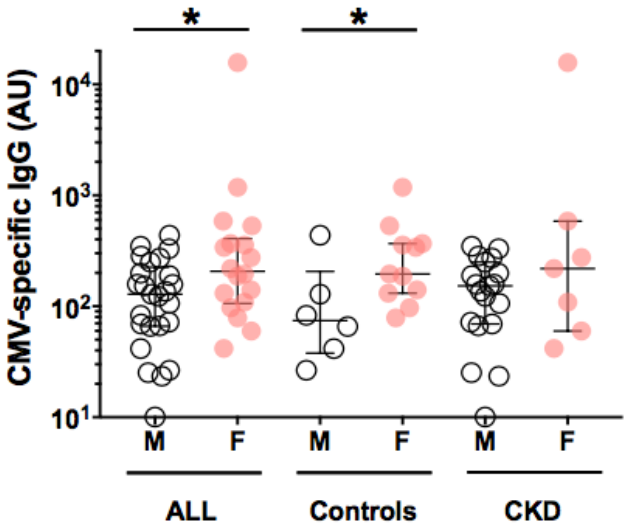


CMV serostatus was the only significant independent predictor of $CD8^+$ proportion of T cells in a linear regression model that also included age, gender and CKD status (p=0.005). However, female gender was a significant predictor of $CD4^+$ proportion of T cells (p=0.03) in addition to CMV serostatus (p=0.003) when the same linear regression

analysis was applied. Although a slightly higher proportion of patients with CKD were CMV seropositive than controls, there were no significant differences in the proportions of male and female participants that were CMV seropositive (n=26 for males, n=18 for females - 72% for both genders, Fisher's exact 2-tailed p=1.0). However, CMV seropositive female participants had significantly higher CMV-specific IgG titres than seropositive males (Mann Whitney 2-tailed p=0.03; Figure 5-5) and this may explain the above linear regression findings.

Figure 5-5 CMV-specific IgG titres in CMV seropositive individuals split by gender.

Rose colour represents data from females. Error bars show median and IQR. *denotes Mann-Whitney 2-tailed p<0.05.



The outcomes of the above multivariate analyses were not altered by the substitution of CKD status by CCI as a more comprehensive multi-morbidity measure.

5.3 Latent CMV infection and female gender significantly influences T_{EMRA} expansion in older adults with and without chronic disease

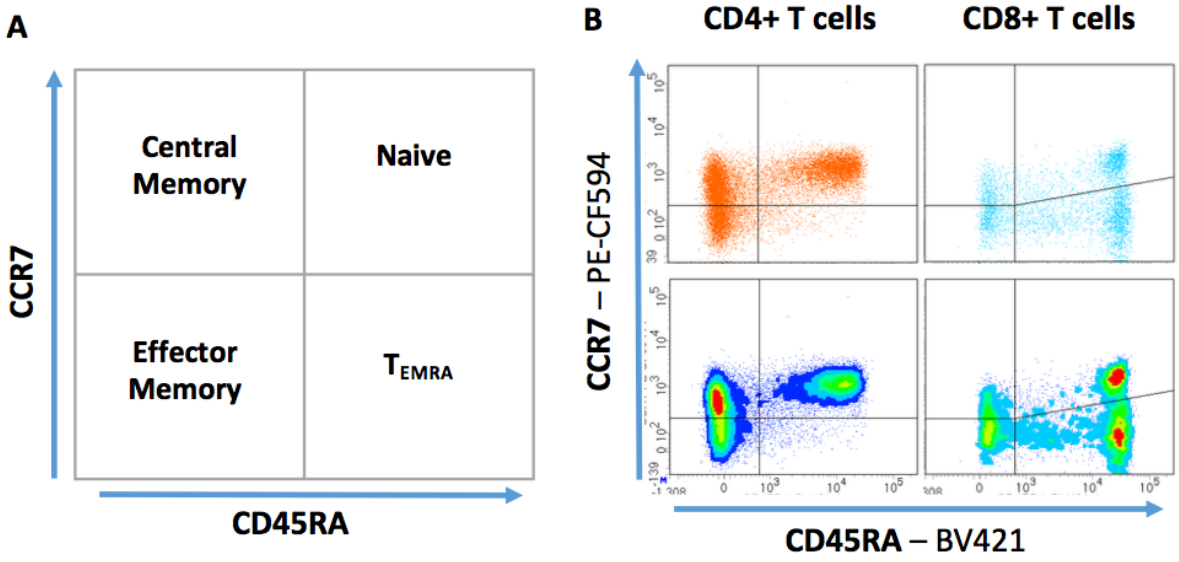
Several strategies have previously been employed to assess immune ageing of the T cell compartment. These include evaluation of naïve versus memory subsets (surrogates for antigen experience) using expression of the surface markers CCR7 and CD45RA (297), the loss of co-stimulatory molecules e.g. CD28 and CD27 (298) and gain of surface markers associated with replicative senescence e.g. CD57 and KLRG1 (17). Each of these strategies were, in turn, employed in this study to assess T cell "ageing" in patients with CKD.

Figure 5-6 shows the gating strategy for identification of naïve/memory populations of T cells. Here, $CCR7^{+}CD45RA^{+}$ cells were deemed "naïve", $CCR7^{+}CD45RA^{+}$ - "central memory", $CCR7^{-}CD45RA^{-}$ - "effector memory" and $CCR7^{-}CD45RA^{+}$ - "terminally differentiated effector memory T cells" or T_{EMRA}.

No significant differences were seen in the proportions of naïve and memory subsets in the CD4+ or CD8+ T cell compartments between controls and patients with CKD (Figure 5-7).

Figure 5-6 Representative gating strategy to identify naïve/memory T cell subsets.

Naïve and memory T cell populations were defined by CCR7 and CD45RA expression as shown in A. CD4+ and CD8+ T cells were identified as in Figure 5-1, then gated as shown in B (top panels show representative scatter plots, bottom panels show density plots). CD8+ and CD4+ T cells had different patterns of expression of CCR7, hence the angle of the lower bound of the CD8 naïve subset is different to CD4 to enable accurate gating.



Markers of renal impairment/damage (eGFR, ACR) were not significantly associated with any of these naïve/memory subsets, except for central memory proportions of $CD8^+$ T cells, which increased with declining eGFR (see Figure 5-8). Measures of multimorbidity (CCI, medication burden), glycaemic control (HbA1c) or inflammation (hsCRP) were also not associated with any of these naïve/memory T cell populations.

Figure 5-7 Naïve/memory T cell subsets in SONIC study participants.

Naïve/memory T cell populations of CD4+ (A,C,E,G) and CD8+ (B,D,F,H) T cells shown for controls and patients with CKD based on CCR7 and CD45RA expression. Proportions of total CD4/8 T cells shown. Error bars show median and IQR.

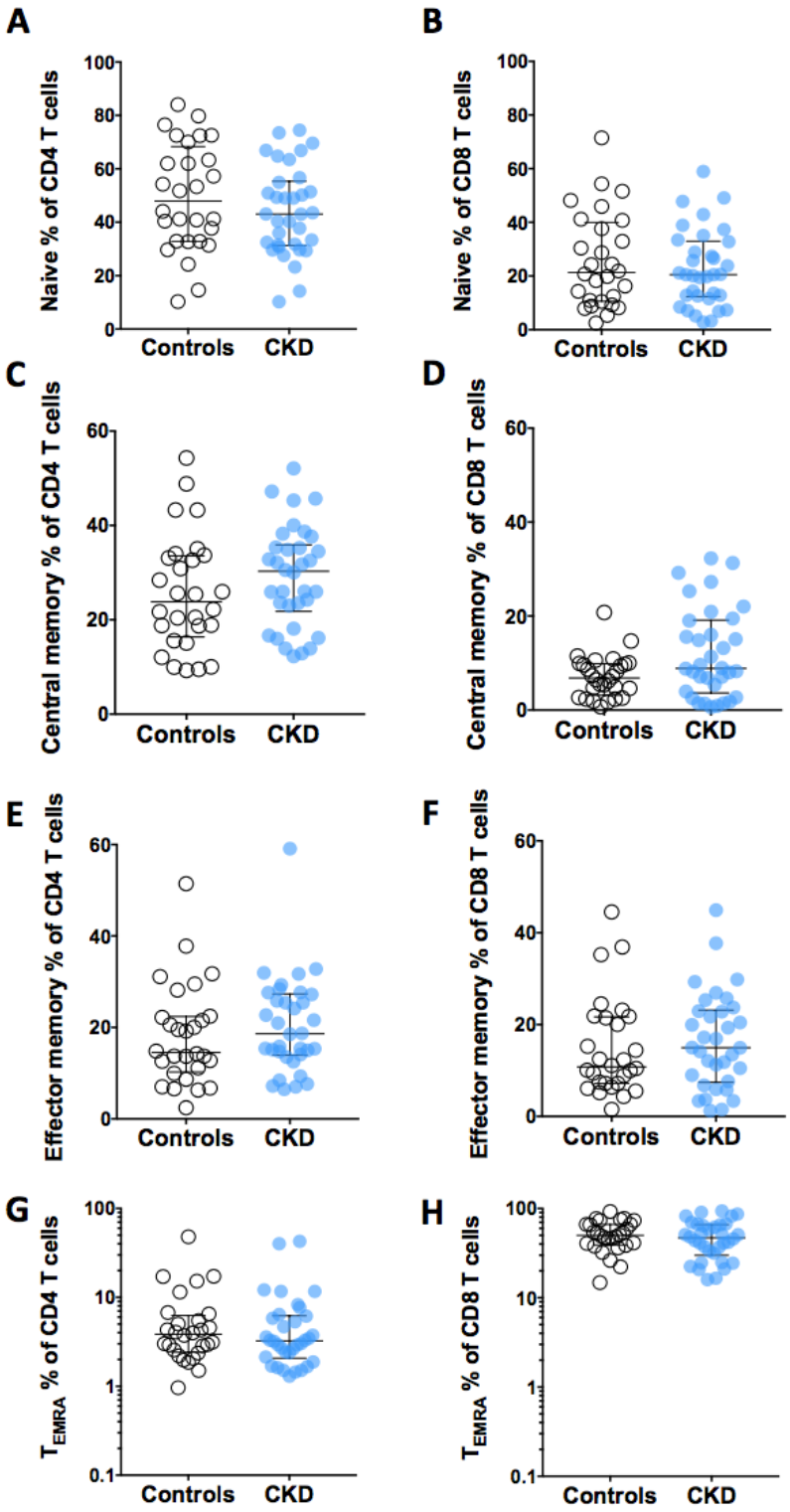
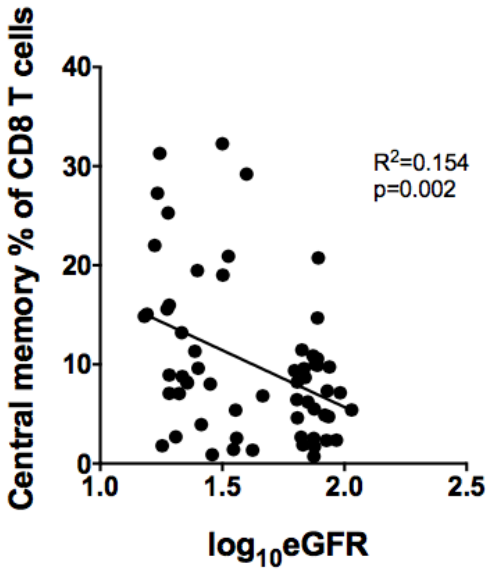


Figure 5-8 Relationship between eGFR and central memory proportion of CD8+ T cells.

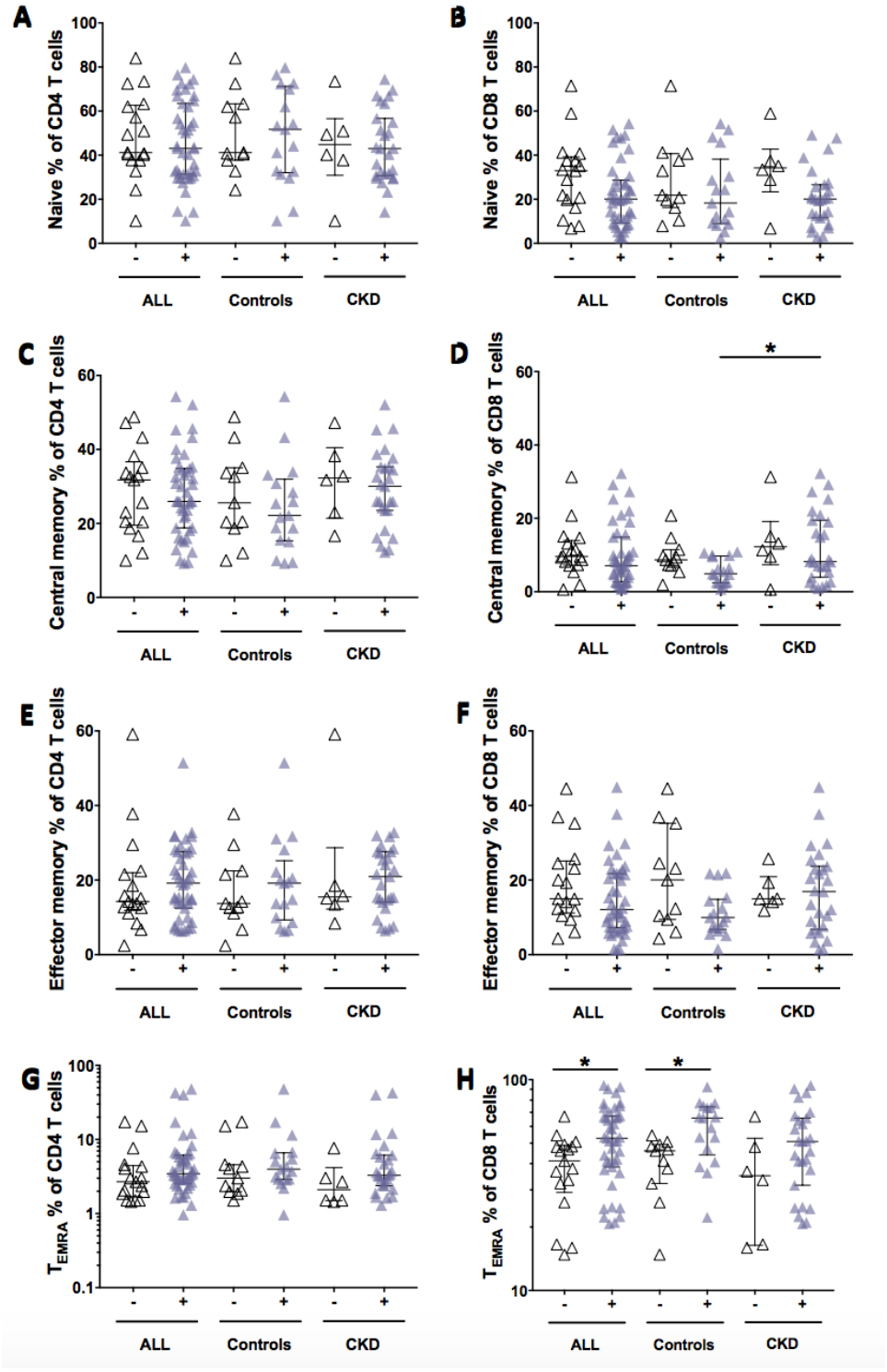
Linear regression R^2 and p value shown. MDRD eGFR shown in plot, but closely approximates CKD-EPI eGFR. P value <0.05 considered significant.



However, CMV-specific IgG titre significantly correlated with the size of the T_{EMRA} proportion of both $CD4^+$ and $CD8^+$ T cells across the whole study (Spearman's coefficient 0.27, p=0.035 and Spearman's coefficient 0.26, p=0.045, respectively). When both controls and patients with CKD were considered together, CMV seropositive individuals had significantly higher T_{EMRA} proportions (unpaired t test 2-tailed p=0.01) and slightly lower naïve proportions of $CD8^+$ T cells than seronegatives (Mann Whitney 2-tailed p=0.06). The $CD4^+$ T_{EMRA} population was also slightly larger in CMV seropositive individuals than in seronegatives (Mann Whitney 2-tailed p=0.12).

Figure 5-9 CMV effect on naïve/memory T cell subsets in SONIC study participants.

Naïve/memory subsets of CD4+ (A,C,E,G) and CD8+ (B,D,F,H) compared between CMV seronegative (-) and seropositive (+) individuals. Lilac colour represents data from CMV seropositive individuals. Error bars show median and IQR. *denotes $p<0.05$



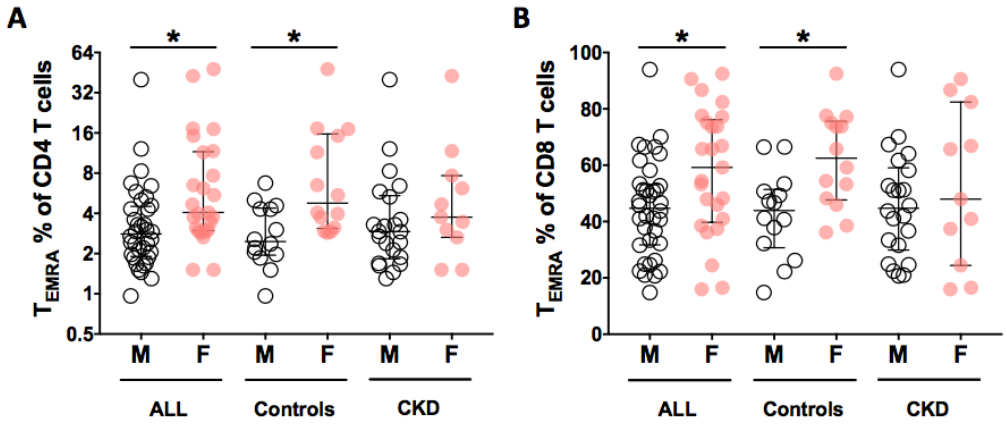
Interestingly, the central memory proportion of $CD8^+$ T cells was significantly larger in CMV seropositive patients with CKD compared to CMV seropositive controls (Mann Whitney 2-tailed p=0.04), mirroring the correlation seen with eGFR. This suggests that renal impairment may have an independent effect on expansion of the central memory population of $CD8^+$ T cells.

CMV serostatus and gender were both significant predictors of the size of the T_{EMRA} proportion of $CD8^+$ T cells (p=0.004 and 0.01, respectively) in a linear regression model that also included age and CKD status. However, when this model was applied to T_{EMRA} proportions of $CD4^+$ T cells, gender emerged as a significant predictor independent of CMV serostatus (p=0.01). Female study participants generally had higher proportions of T_{EMRA} cells in both CD4+ and CD8+ T cell compartments than males (Figure 5-10). Although CMV-specific IgG titres were significantly greater in female participants than males (Figure 5-5), gender remained a significant predictor of T_{EMRA} proportions in both $CD4^+$ and $CD8^+$ T cells in CMV seropositive individuals, independent of age, CKD status and CMV-specific IgG titre. This suggests an independent gender effect on these T cell populations, the biological significance of which is unclear.

No significant predictors of central memory proportions of $CD8^+$ T cells were identified in a linear regression model that included age, gender, CKD status and CMV serostatus, either when all study participants or only CMV seropositive individuals were considered. This was not altered by substitution of CCI for CKD status as a more comprehensive measure of multimorbidity.

Figure 5-10 Gender effect on CD4+ (A) and CD8+ (B) T_{EMRA} populations.

Rose colour represents data from females. Error bars show median and IQR. *denotes p<0.05.

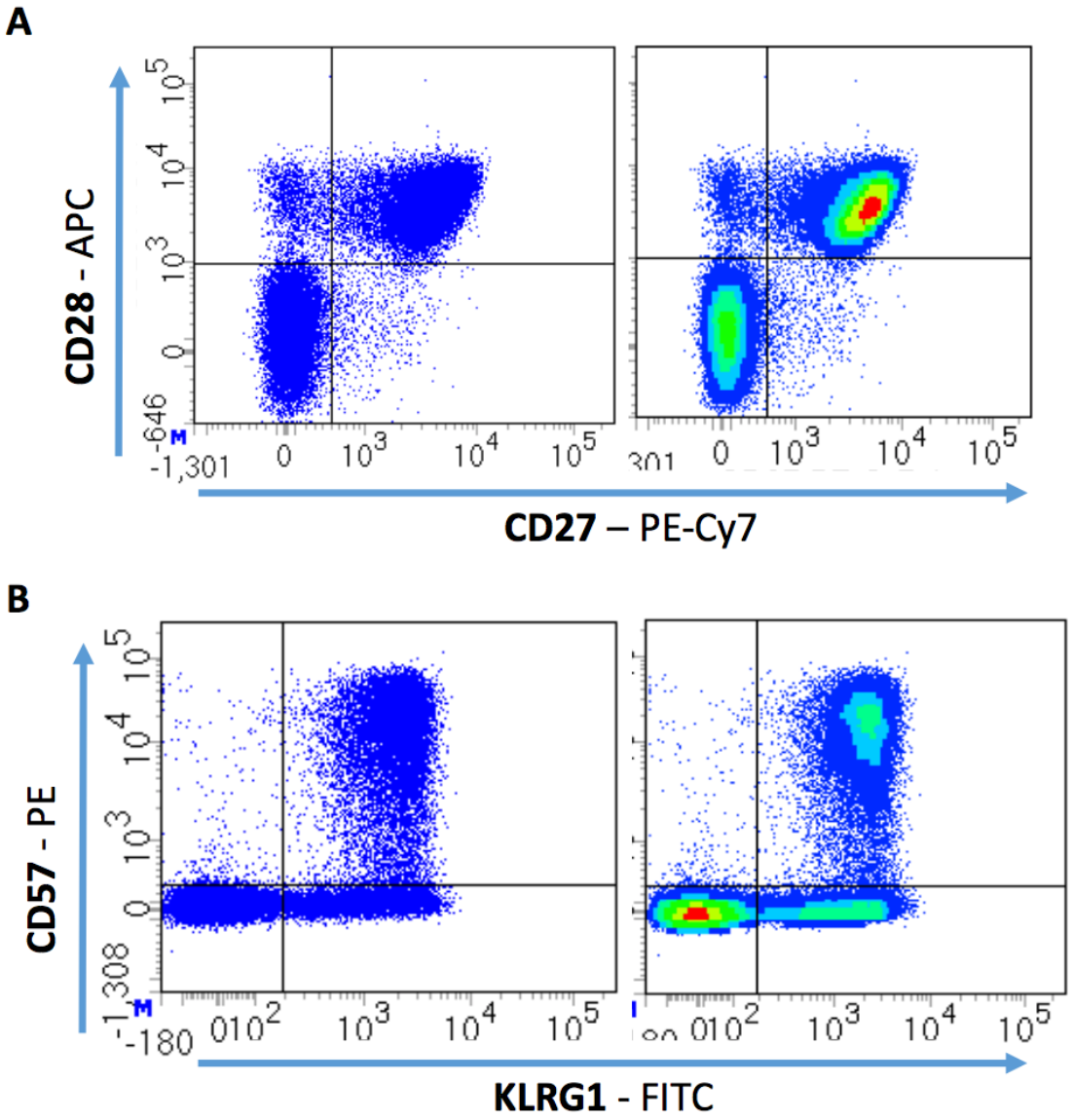


5.4 Latent CMV infection and magnitude of humoral CMV-specific response determine "senescent" T cell phenotype in older adults with and without chronic disease

The loss of surface expression of CD27 and/or CD28 is associated with terminally differentiated T cells. This, together with gain of surface CD57 and/or KLRG1 expression, has been associated both with chronological immune ageing (17) and with latent CMV infection (88). Together, these 4 surface markers are associated with replicative senescence in T cells (17) and phenotypes using combinations of these markers will henceforth be referred to as "senescence"-associated, for brevity. These populations were assessed in the SONIC study, with representative gating shown in Figure 5-11.

Figure 5-11 Gating strategy to identify "senescence"-associated T cell subtypes.

Representative gating shown for CD27/28 (A) and CD57/KLRG1 (B) after CD4/8 T cells were identified as in Figure 5-1. Left panels show representative scatter plots, right panels show density plots.



No significant differences were seen between patients with CKD and controls in the proportions of $CD4^+$ or $CD8^+$ T cells either lacking CD28 alone or in combination with CD27, or the proportions expressing both CD57 and KLRG1 (Figure 5-12 A,C,E,G and Figure 5-13 A,C,E,G).

Figure 5-12 CD4+ T cell "senescence" phenotyping.

Comparisons between controls and patients with CKD (A,C,E,G) and impact of latent CMV (B,D,F,H) – here +/- denotes CMV seropositive/negative. Error bars show median and IQR. *denotes p<0.05

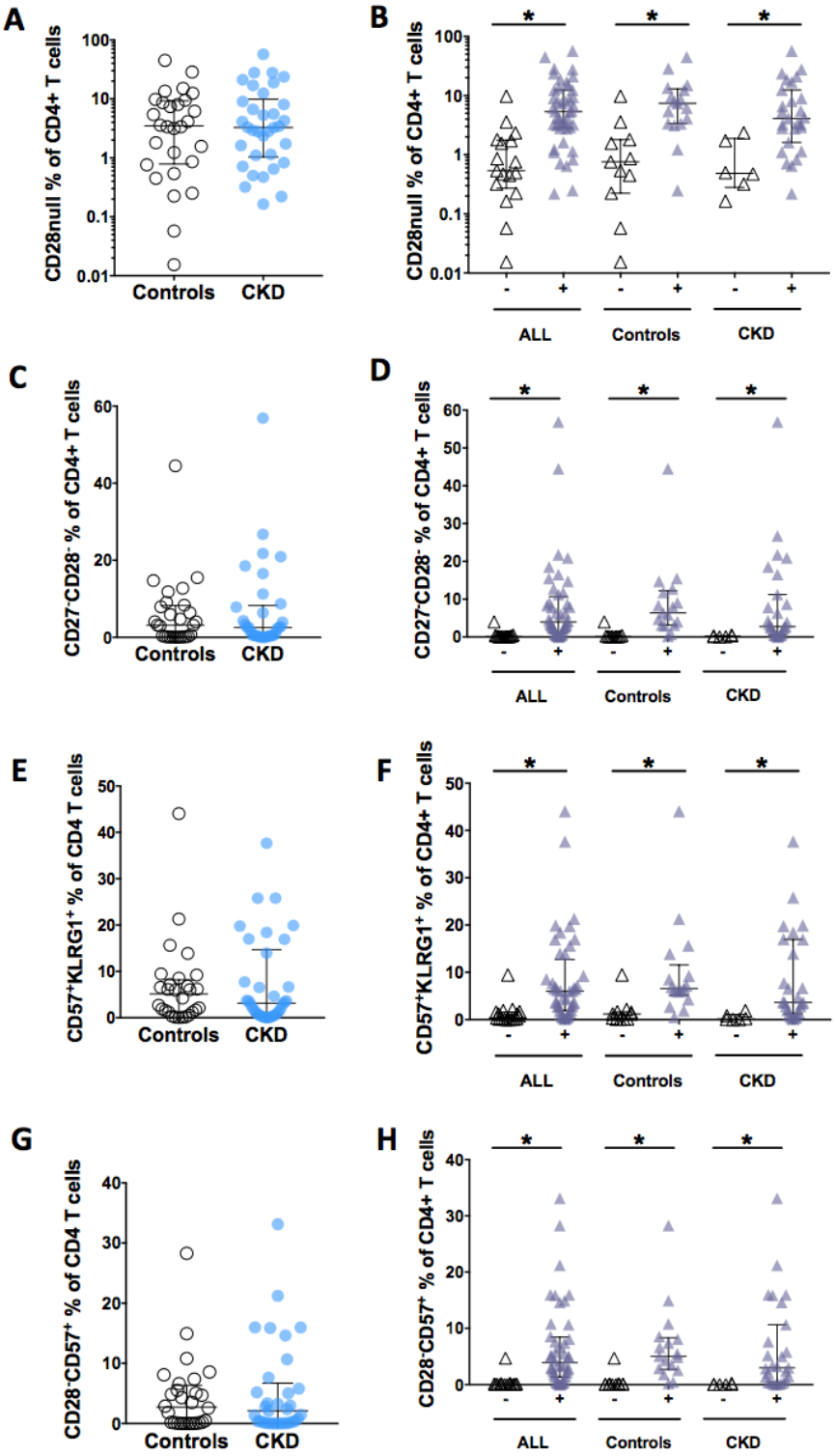
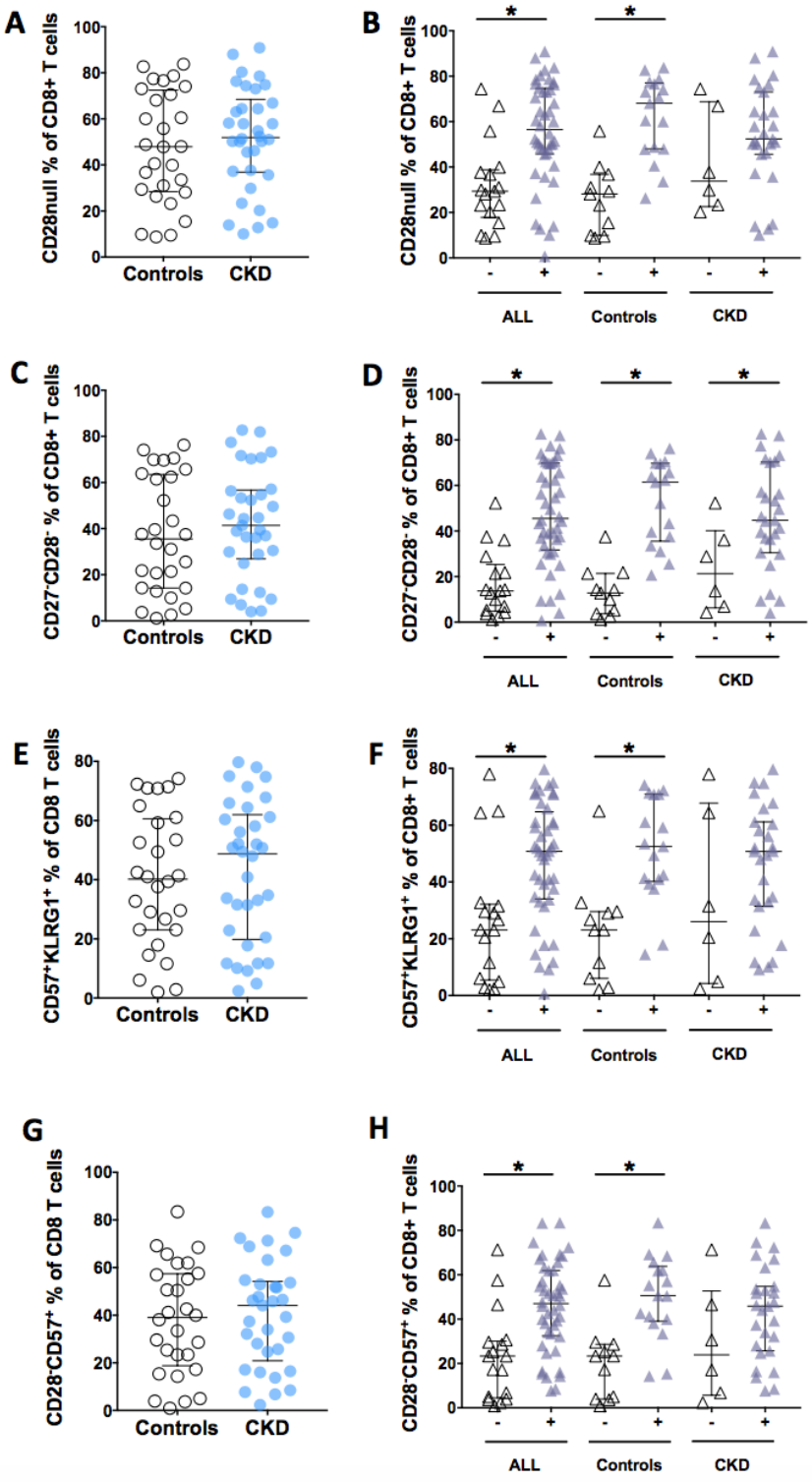


Figure 5-13 CD8+ T cell "senescence" phenotyping.

Comparisons between controls and patients with CKD (A,C,E,G) and impact of latent CMV (B,D,F,H) – here +/- denotes CMV seropositive/negative. Error bars show median and IQR. *denotes $p<0.05$.



However, CMV seropositive individuals had significantly higher proportions of $CD28^{null}$, $CD27^{-}CD28^{-}$, $CD57^{+}KLRG1^{+}$ and $CD28^{-}CD57^{+}$ cells in both CD4+ and CD8+ T cell compartments (Figure 5-12 and Figure 5-13). Interestingly, CMV-specific IgG titre in seropositive individuals was significantly associated with all $CD4^{+}$ "senescence"-associated phenotypes (Figure 5-14), but none in the $CD8^{+}$ T cell compartment (Figure 5-15), although the $CD27^{-}CD28^{-}$ proportion of $CD8^{+}$ T cells approached significance (p=0.07).

Figure 5-14 Associations between CMV-specific IgG titre in CMV seropositive individuals and CD4+ T cell "senescence"-associated phenotypes.

Relationships between CMV-specific IgG titre and A: $CD28^{null}$, B: $CD27^{-}CD28^{-}$, C: $CD57^{+}KLRG1^{+}$, D: $CD28^{-}CD57^{+}$ % of $CD4^{+}$ T cells. Linear regression statistics shown. P <0.05 considered significant.

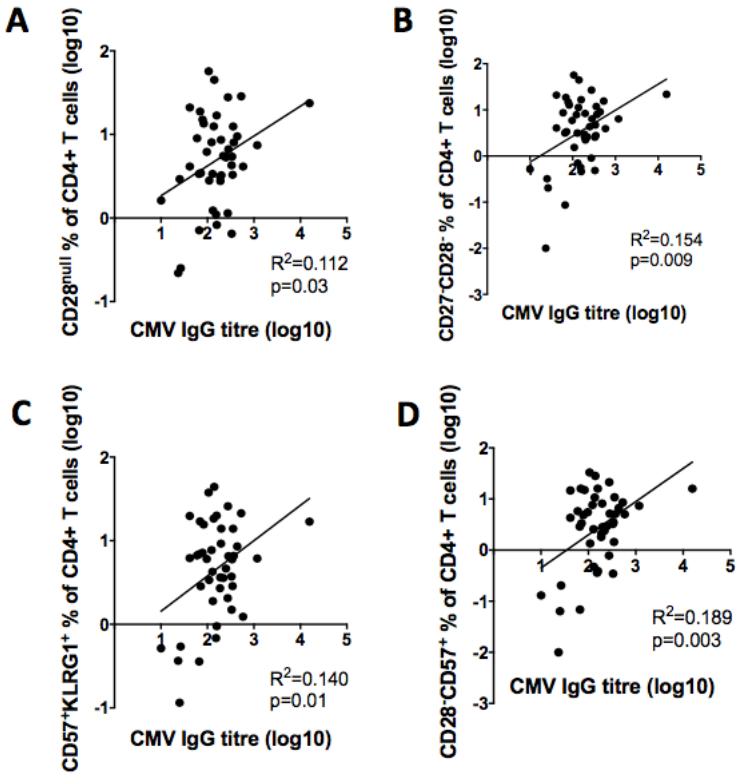
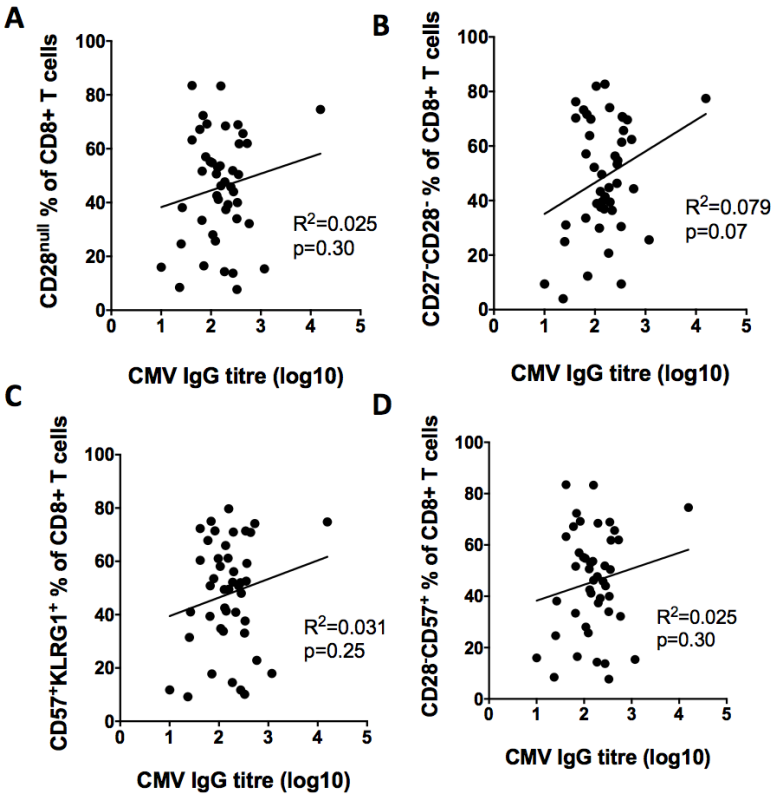


Figure 5-15 Associations between CMV-specific IgG titre in CMV seropositive individuals and CD8+ T cell "senescence"-associated phenotypes.

Relationships between CMV-specific IgG titre and A: $CD28^{null}$, B: $CD27^{-}CD28^{-}$, C: $CD57^{+}KLRG1^{+}$, D: $CD28^{-}CD57^{+}$ % of $CD8^{+}$ T cells. Linear regression statistics shown. P <0.05 considered significant.



There were no significant relationships between proportions of "senescence"-associated $CD4^{+}$ or $CD8^{+}$ T cell subsets and eGFR, ACR, HbA1c or hsCRP. However, several significant associations were seen between measures of multimorbidity (CCI and medication burden) and $CD8^{+}$, but not $CD4^{+}$ "senescence"-associated subsets, although the proportion of $CD27^{-}CD28^{-}$ $CD4^{+}$ T cells approached significance for both multimorbidity parameters (Table 5-1).

Table 5-1 Relationships between measures of multimorbidity and "senescence"-associated T cell subsets.

Correlations between multimorbidity measures (top row) and "senescence"-associated T cell subsets (first column). Spearman R and 2-tailed p shown and highlighted in bold if $p<0.05$ (deemed significant).

	CCI	Medication burden
CD4+		
CD28null %	R=0.13 p=0.33	R=0.17 p=0.19
CD27-CD28- %	R=0.23 p=0.08	R=0.23 p=0.08
CD57+KLRG1+ %	R=0.19 p=0.15	R=0.18 p=0.17
CD28-CD57+ %	R=0.18 p=0.19	R=0.16 p=0.26
CD8+		
CD28null %	***R=0.27*** ***p=0.04***	***R=0.31*** ***p=0.02***
CD27-CD28- %	***R=0.28*** ***p=0.03***	***R=0.32*** ***p=0.01***
CD57+KLRG1+ %	R=0.23 p=0.08	***R=0.27*** ***p=0.04***
CD28-CD57+ %	R=0.21 p=0.10	***R=0.26*** ***p=0.05***

Multivariate analysis confirmed CMV serostatus as a significant predictor of all "senescence"-associated $CD8^+$ T cell phenotypes independent of age, gender and CKD status (Table 5-2). This was not altered with substitution of CKD status by CCI or medication burden. CMV serostatus was also a significant predictor of all "senescence"-associated $CD4^+$ T cell phenotypes, but female gender was a significant co-factor in predicting expansions of $CD28^-$ and $CD27^-CD28^-$ subsets. As with the $CD8^+$

compartment, these relationships were not altered by the substitution of CKD status by CCI or medication burden.

Table 5-2 Multivariate analysis to identify predictors of T cell "senescence"-associated subsets.

Linear regression p values shown for predictor variables of interest (first column) for individual "senescence"-associated T cell subsets (top row). Model included age, gender, CKD status and CMV serostatus. P value <0.05 considered significant (highlighted in bold).

All participants	**CD4 T cells**				**CD8 T cells**			
	$CD28^{null}$ %	**CD27-CD28- %**	**CD57+ KLRG1+ %**	**CD28-CD57+ %**	**$CD28^{null}$ %**	**CD27-CD28- %**	**CD57+ KLRG1+ %**	**CD28-CD57+ %**
Gender (M/F)	***0.009***	***0.009***	0.49	0.07	0.36	0.30	0.36	0.46
CMV serostatus (+/-)	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***	***0.0003***	***<0.0001***	***0.004***	***0.001***

As female CMV seropositive individuals in this study had a significantly higher CMV-specific IgG titres (Figure 5-5) and the magnitude of this titre was significantly associated with expansions of $CD4^+$ T cell "senescence"-associated subsets, the linear regression results may simply reflect a CMV-associated effect. However, neither CMV-specific IgG or gender were significant predictors of any of these subsets in CMV seropositive individuals only in a linear regression model that also included age, gender and CKD status.

These findings suggest that latent CMV infection defines the "senescence"-associated phenotype of both $CD4^+$ and $CD8^+$ T cell compartments in older adults with and without chronic disease, independent of the degree of multimorbidity or, indeed, renal impairment.

5.5 CKD significantly affects $CD4^+$ helper and regulatory T cell populations, independent of latent CMV infection

I evaluated the proportions of $CD4^+$ T cell helper and regulatory subtypes in a sample of study participants (limited by volume of blood collected and assay failure; n=18 per group for both patients with CKD and controls) based on surface chemokine receptor expression, which has been shown previously to approximate subtyping by transcription factor analysis and cytokine secretion (15, 16). As shown in Figure 5-16, $CCR4^-CCR6^-CXCR3^+$ were defined as "Th1-like" CD4 T cells, $CCR4^+CCR6^-CXCR3^-$ as "Th2-like", $CCR4^+CCR6^+CXCR3^-$ as "Th17-like" and $CCR4^-CCR6^+CXCR3^+$ as "Th1Th17-like" (an atypical subset of Th1 cells previously shown to produce both IFN-γ and IL-17) (16). "Tfh-like" CD4 T cells were identified by expression of CXCR5 (B cell follicle-homing receptor) as previously described (15).

Regulatory CD4 T cells (T_{regs}) were identified by expression of the surface marker CD25 and the lineage-specific intracellular transcription factor - FoxP3 - by two different gating strategies as shown in Figure 5-17. Initially, T_{regs} were defined as the $CD25^+FoxP3^+$ population after gating on $CD4^+$ T cells (Figure 5-17 C) as per methods described in references (299-302) – hereafter referred to as $CD25^+FoxP3^+$ T_{regs}. During flow cytometry panel optimisation this population of cells was consistently negative for CD127 and this marker was therefore not used in subsequent analysis. Several authors have defined a more stringent gating strategy for T_{regs} (231, 303, 304), which I have also employed as shown in Figure 5-17D to enable wider comparisons with published data. Here, $CD4^+$ T cells with the highest expression of CD25 were selected ($CD25^{high}$) and then assessed for intracellular expression of FoxP3 – hereafter referred to as $CD25^{high}FoxP3^+$ T_{regs}.

Figure 5-16 Representative gating to identify "helper" CD4+ T cell sub-populations by surface chemokine receptor expression.

Density plots shown. CD4 T cells were identified as per Figure 5-1 and "Tfh-like" CD4+ T cells were defined by surface expression of CXCR5 (A). Total CD4 T cells were then evaluated for surface CCR4 and CCR6 expression. Each quadrant from this gate was then evaluated for surface CXCR3 expression to define "helper" CD4+ T cell phenotypes as shown in B.

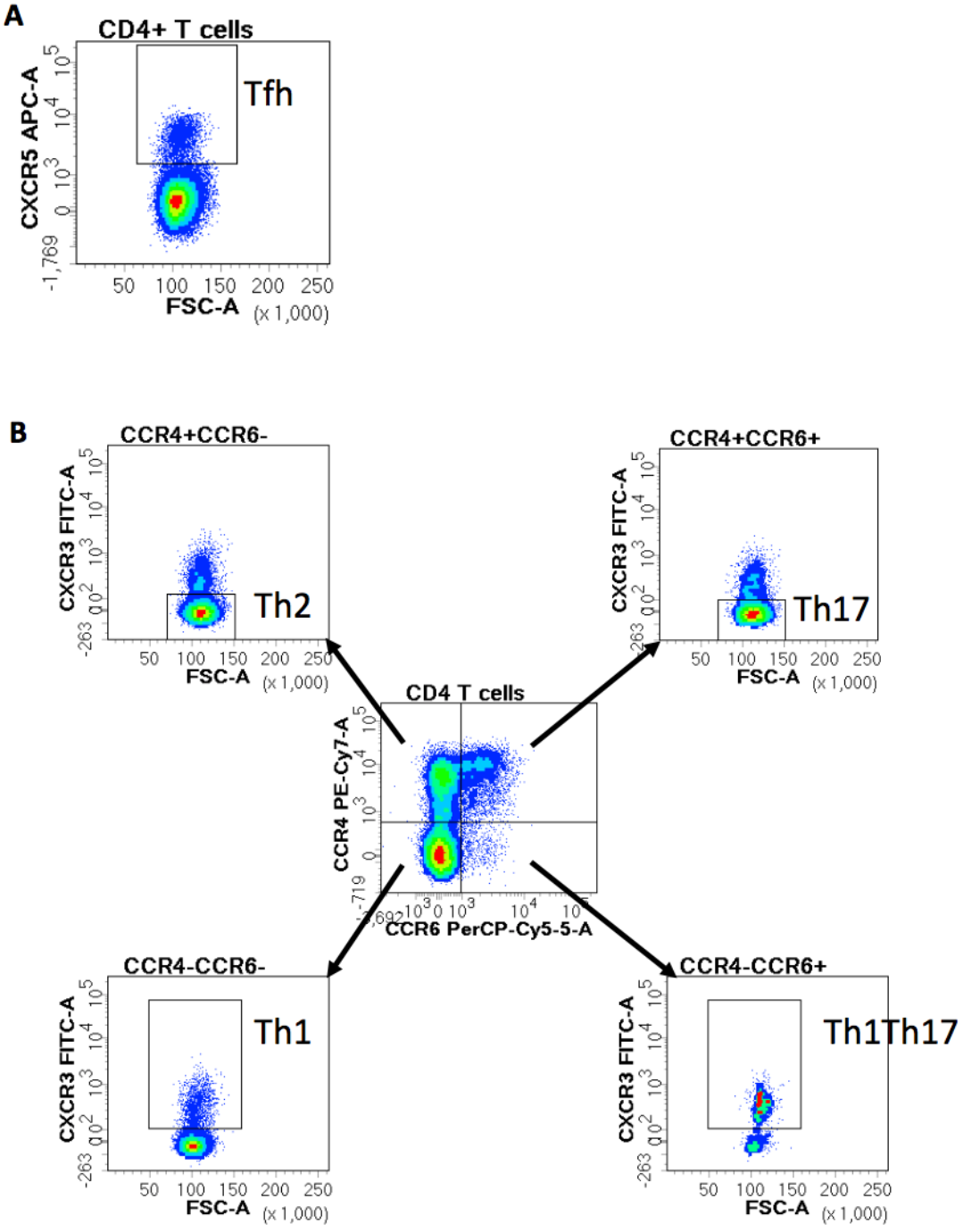
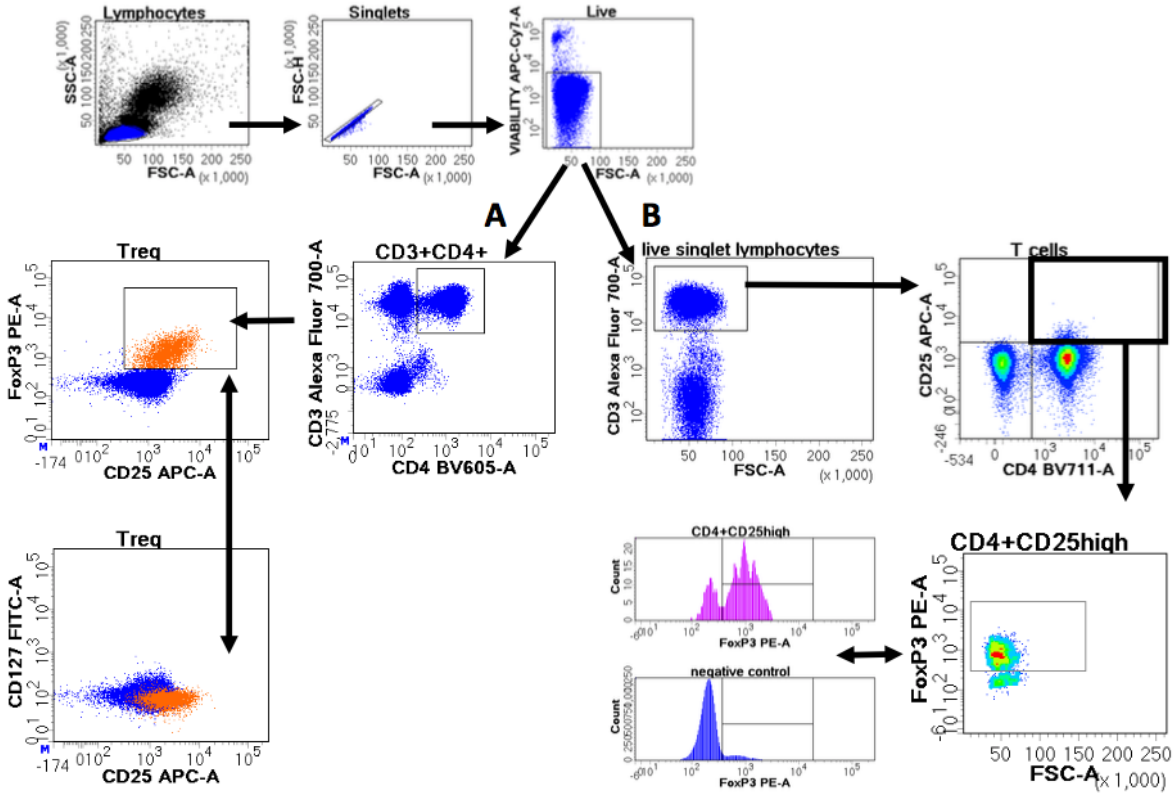


Figure 5-17 Representative gating to identify regulatory T cells.

Scatter plots shown unless stated. Live singlet lymphocytes were identified based on forward and side scatter characteristics and viability stain (APC-Cy7). $CD4^+$ T cells were identified and then evaluated for expression of CD25 and FoxP3 in two ways: A - $CD25^+FoxP3^+$ (with representative CD127 expression plot demonstrating lack of CD127 expression in this population); B - $CD25^{high}FoxP3^+$ density plots with histograms representing FoxP3 expression in patient samples and negative assay control.



Patients with CKD had similar proportions of "Th1-", "Th17-", "Th1Th17-" and "Tfh-like" CD4 T cells to controls, but a significantly greater proportion of "Th2-like" CD4 T cells (Figure 5-18), independent of CMV serostatus (Figure 5-19 B).

Figure 5-18 Helper CD4+ T cell subtypes in SONIC study participants.

"Th1-like" (A), "Th17-like" (B), "Th1Th17-like" (C), "Tfh-like" (D) and "Th2-like" (E) subtypes as proportions of total CD4+ T cell population – comparison between controls and patients with CKD. Representative flow density plots from control and CKD individuals shown in panel F – for illustrative purposes, "Th2-like" population is gated as CCR4+ from CXCR3-CCR6- population. Error bars show median and IQR. *denotes $p<0.05$

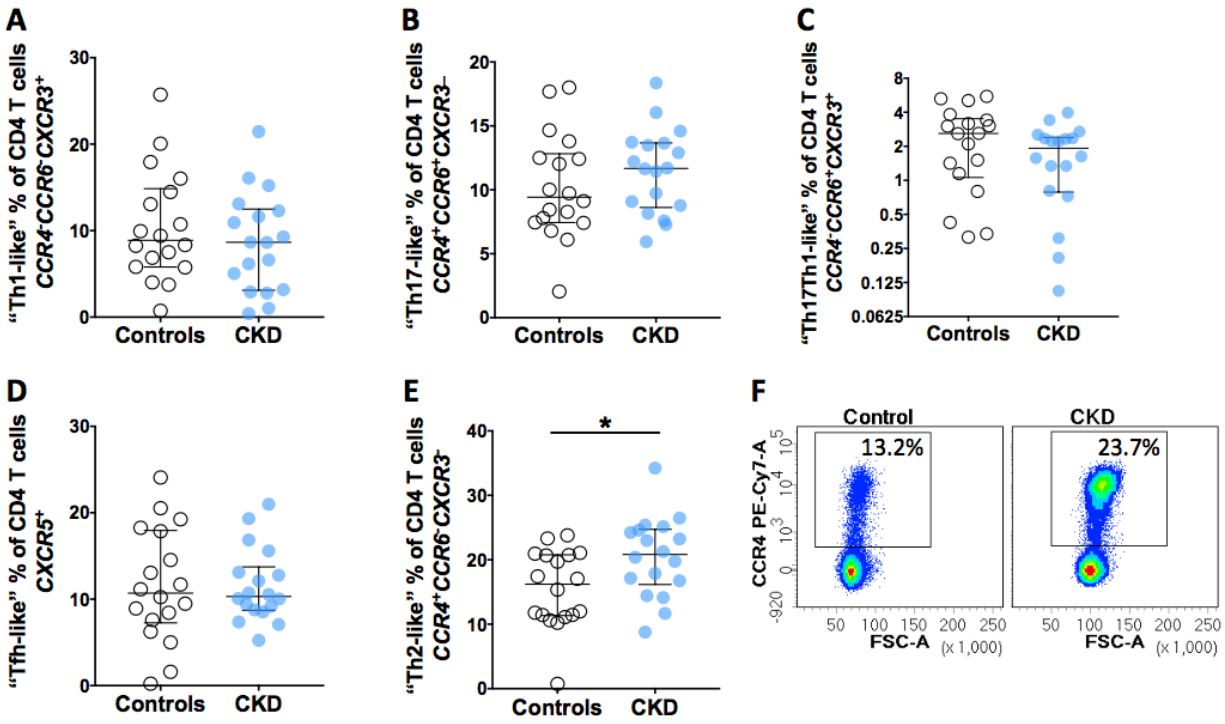
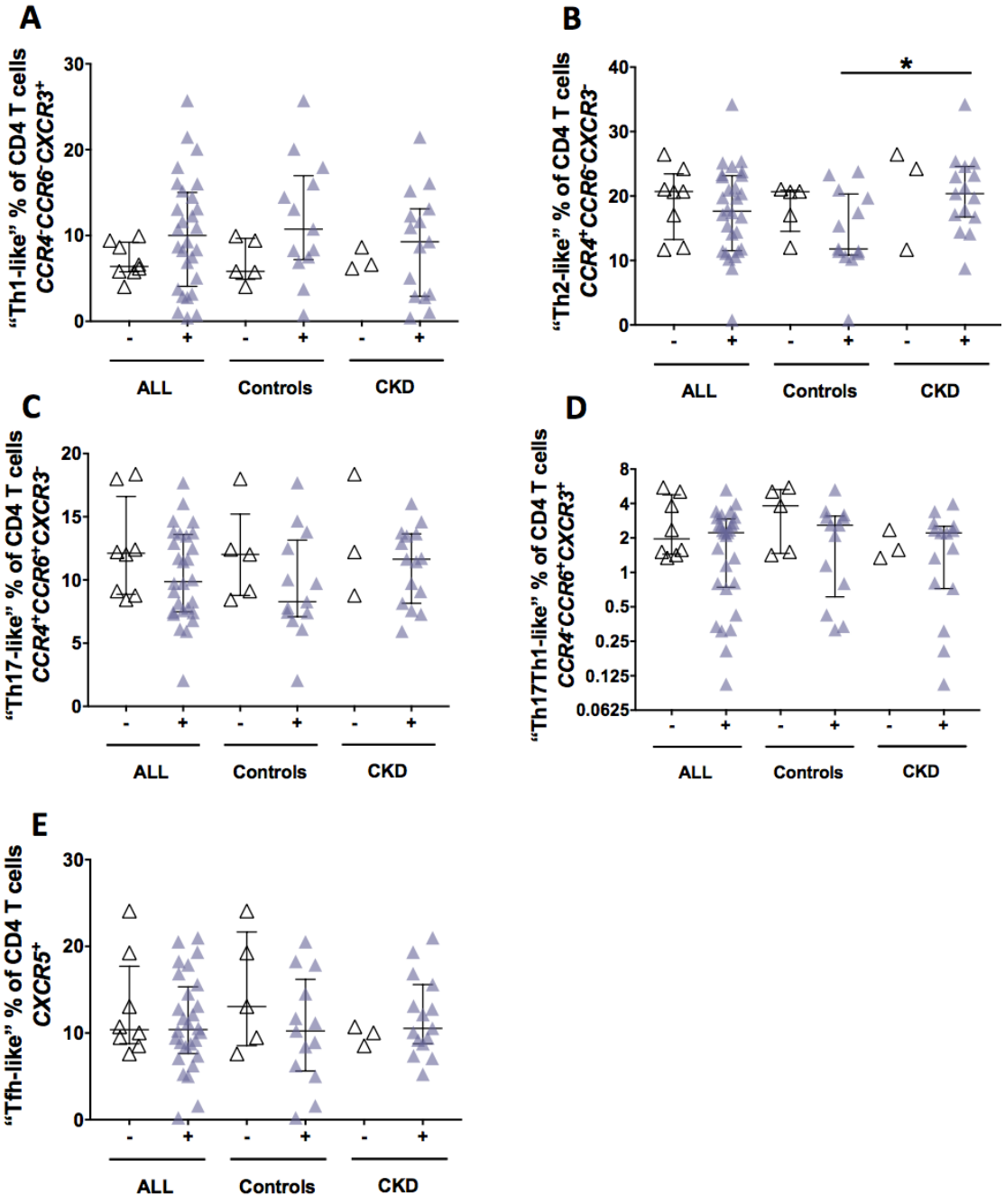


Figure 5-19 Effect of latent CMV on CD4+ T cell helper subtypes.

"Th1-like" (A), "Th2-like" (B), "Th17-like" (C), "Th1Th17-like" (D) and "Tfh-like" (E) subsets shown as proportions of total CD4+ T cells, compared between CMV seronegative (-) and seropositive (+) individuals. Error bars show median +/- IQR. *denotes Mann Whitney 2-tailed $p<0.05$.



Proportions of "Th2-" and "Th1-like" CD4 T cells had a significant reciprocal relationship (Pearson's correlation coefficient -0.52, 2-tailed p=0.001), when controls and patients with CKD were considered together. Interestingly, proportions of "Th2-like" cells also positively correlated with proportions of "Th17-" and "Tfh-like" CD4 T cells (Pearson's coefficient 0.63, 2-tailed p<0.0001 and Pearson's coefficient 0.38, 2-tailed p=0.02, respectively). Expansions of the "Th2-like" phenotype in the $CD4^+$ compartment were associated with a reduction in eGFR (Pearson's correlation coefficient -0.36, 2-tailed p=0.03 for both MDRD and CKD-EPI eGFR), but less so with increasing proteinuria (Pearson's coefficient 0.31, p=0.07 for baseline ACR). No significant associations were seen between any of the $CD4^+$ helper subtypes and CCI, medication burden, HbA1c, hsCRP or CMV-specific IgG titre.

CKD status was a significant predictor of "Th2-like" proportion of $CD4^+$ T cells, independent of age, gender and CMV serostatus (p=0.02). The linear regression model fit was not improved by the addition of the collinear variables CCI or medication burden, suggesting that renal dysfunction alone is important in describing the expansion of this cell population. No significant predictors were identified using this model for any of the other CD4+ helper subtype proportions.

Patients with CKD had significantly greater proportions of T_{reg} $CD4^+$ T cells than controls, independent of gating strategy ($CD25^+FoxP3^+$ T_{regs} or $CD25^{high}FoxP3^+$ T_{regs}) and CMV serostatus (Figure 5-20). Expansions of T_{reg} CD4 T cells were significantly associated with expansions of "Th2-like" CD4 T cells in this study (Figure 5-21), suggesting a common process driving expansions of both of these populations. Indeed, expansions of T_{regs} were significantly associated with reducing eGFR (Pearson's coefficient -0.39, 2-tailed p=0.02 for both gating strategies and MDRD eGFR and Pearson's coefficient -0.35, 2-tailed

p≤0.04 for both gating strategies and CKD-EPI eGFR) and increasing proteinuria (Pearson's coefficient 0.48, 2-tailed p<0.01 for both gating strategies and baseline ACR). No significant associations were seen between T_{reg} proportion of $CD4^+$ T cells (using either gating strategy) and HbA1c, CMV-specific IgG titre or hsCRP. Interestingly, $CD25^{high}FoxP3^+$ T_{reg} proportion of $CD4^+$ T cells was significantly associated with both CCI and medication burden (Spearman R=0.37, p=0.03 and R=0.47, p=0.004, respectively), but $CD25^+FoxP3^+$ T_{reg} proportion only with medication burden (Spearman R=0.38, p=0.02).

CKD status was the only significant predictor of both $CD25^+FoxP3^+$ T_{reg} or $CD25^{high}FoxP3^+$ T_{reg} proportion of $CD4^+$ T cells in a linear regression model that also included age, gender and CMV serostatus (p=0.02 and 0.004 respectively). The addition of the collinear multimorbidity variable CCI (but not medication burden) to CKD status did improve model fit, suggesting that renal impairment and multimorbidity both have an impact on T_{reg} populations.

Figure 5-20 CD4+ regulatory T cell subtypes.

$CD25^{+}FoxP3^{+}$ Tregs: A, B, C and $CD25^{high}FoxP3^{+}$ Tregs: D, E, F, shown as proportions of total CD4+ T cell population – comparison between controls and patients with CKD (A ,D) with representative flow density plots (C, F) and impact of latent CMV (+ denotes seropositive) (B, E). For panel F, FoxP3 negative total CD4+ population is shown for illustrative purposes – FoxP3 positive gate just on $CD4+CD25^{high}$ cells. Error bars show median +/- IQR. *denotes Mann-Whitney 2-tailed $p<0.05$.

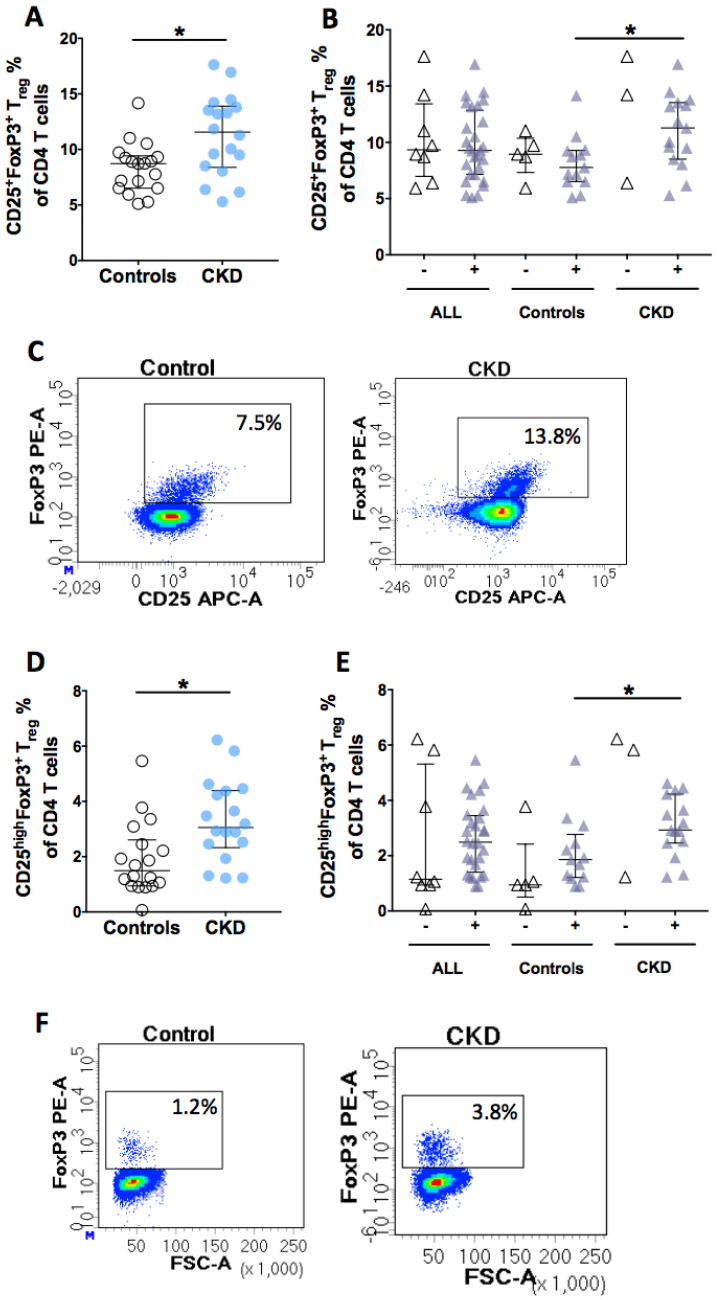
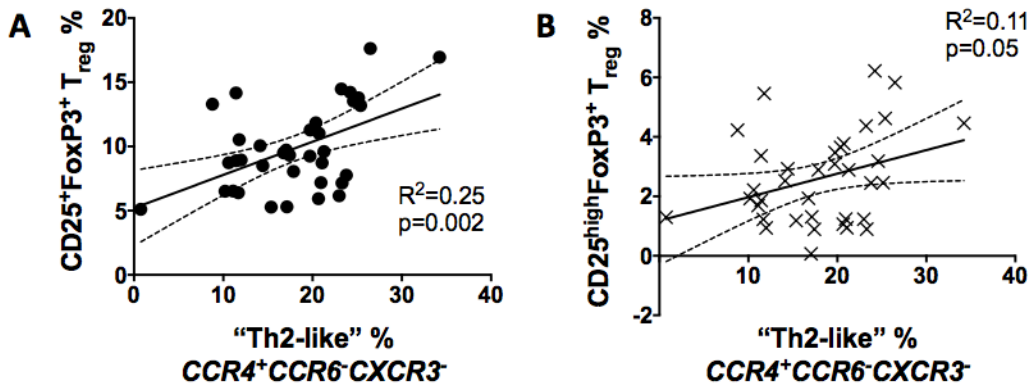


Figure 5-21 Relationships between T_{reg} and "Th2-like" proportions of $CD4^+$ T cells in SONIC study participants.

Linear regression statistics shown for relationships between "Th2-like" proportions of CD4+ T cells and $CD25^+FoxP3^+$ T_{regs} (A) and $CD25^{high}FoxP3^+T_{regs}$ (B). P value <0.05 considered significant.



5.6 T lymphocyte phenotypes associated with vaccine response

Humoral responses to vaccines depend significantly on the function of B lymphocytes and their interaction with T lymphocytes, so it is reasonable to suppose that a difference in T cell phenotype might be seen between individuals with robust and with poor responses to TIV/PPV23.

As described in Chapter 4 (Vaccine response), patients with CKD in this study had largely similar humoral responses to both a T-dependent (TIV) and T-independent (PPV23) vaccine compared to age and gender matched controls. Across the whole study, increasing age and lower baseline HAI titre or serotype-specific anti-PnPS IgG concentration were associated with higher peak responses (ARR) for both TIV and PPV23 vaccines. Previous

PPV23 vaccination status and, unexpectedly, latent CMV infection, were also associated with lower PPV23 ARR.

For brevity and clarity of analysis, in this section I will evaluate relationships between TIV/PPV23 mean ARR (which is largely representative of trends seen with individual antigens) and T lymphocyte profiling as described earlier in this Chapter. As no significant differences in vaccine response were seen between controls and patients with CKD in this study, I have chosen to examine the SONIC study population as a whole to gain insight into potential cellular predictors of vaccine responses in older adults in general (with and without chronic disease). This approach also maximises sample size and allows a more powerful statistical analysis.

5.6.1 Poor TIV responses correlate with expansions of "senescence"-associated T cell subsets in older adults with and without chronic disease.

Relationships between T cell phenotypes analysed in this study and the mean ARR of TIV and PPV23 are summarised in Table 5-3. Higher TIV mean ARR was significantly associated with T cell proportions of total lymphocytes and $CD28^{null}$, $CD27^{-}CD28^{-}$ and $CD57^{+}KLRG1^{+}$ proportions of $CD4^{+}$ T cells (Figure 5-22 A, D-F). A significant inverse correlation was also seen with $CD28^{null}$ and $CD27^{-}CD28^{-}$ proportions of $CD8^{+}$ T cells and this approached significance for $CD57^{+}KLRG1^{+}$ proportions of $CD8^{+}$ T cells (Figure 5-22 G-I). These T cell phenotypes are associated with replicative senescence (17) and have previously been reported to be expanded in older adults (305) and in the context of latent CMV infection (86). Indeed, in this study of older adults with and without chronic disease,

CMV serostatus is a strong predictor of expansions of these populations. Although no significant relationship was seen between TIV mean ARR and CMV-specific IgG titre, the cellular relationships described suggest that latent CMV infection could still play a role in modulating T-dependent antigen responses in older adults.

Table 5-3 Summary of correlations between T cell phenotypes and vaccine responses.

Correlations between T cell phenotypes (first column) and TIV mean ARR (orange) and PPV23 mean ARR (blue). Spearman correlation statistics shown. P value <0.05 considered significant – highlighted in bold.

	TIV mean ARR		PPV23 mean ARR	
	Spearman r	p value	Spearman r	p value
T cell %	0.38	**0.003**	0.01	0.97
$CD4^+$ % of T cells	0.01	0.96	0.01	0.93
$CD8^+$ % of T cells	0.04	0.75	-0.002	0.99
CD4/8 ratio	-0.03	0.80	0.002	0.99
$CD4^+$				
Naïve %	-0.11	0.42	-0.09	0.50
T_{CM} %	0.12	0.36	-0.01	0.94
T_{EM} %	0.03	0.81	0.07	0.59
T_{EMRA} %	-0.14	0.29	0.06	0.64
$CD28^{null}$ %	-0.26	**0.047**	0.01	0.93
$CD27^-CD28^-$ %	-0.27	**0.04**	-0.11	0.40
$CD57^+KLRG1^+$ %	-0.31	**0.02**	-0.07	0.60
"Th1-like" %	0.11	0.55	0.16	0.37
"Th2-like" %	0.03	0.86	-0.10	0.56
"Th17-like" %	0.003	0.99	0.23	0.20
"Th17Th1-like" %	0.19	0.28	0.38	**0.03**
"Tfh-like" %	-0.05	0.77	0.40	**0.02**
T_{REG} %	-0.03	0.87	0.04	0.81
$CD8^+$				
Naïve %	-0.17	0.20	0.08	0.55
T_{CM} %	0.22	0.09	-0.06	0.68
T_{EM} %	0.45	**0.0003**	0.12	0.36
T_{EMRA} %	-0.15	0.25	-0.08	0.54
$CD28^{null}$ %	-0.28	**0.03**	-0.10	0.46
$CD27^-CD28^-$ %	-0.28	**0.03**	-0.11	0.42
$CD57^+KLRG1^+$ %	-0.25	0.06	-0.06	0.65

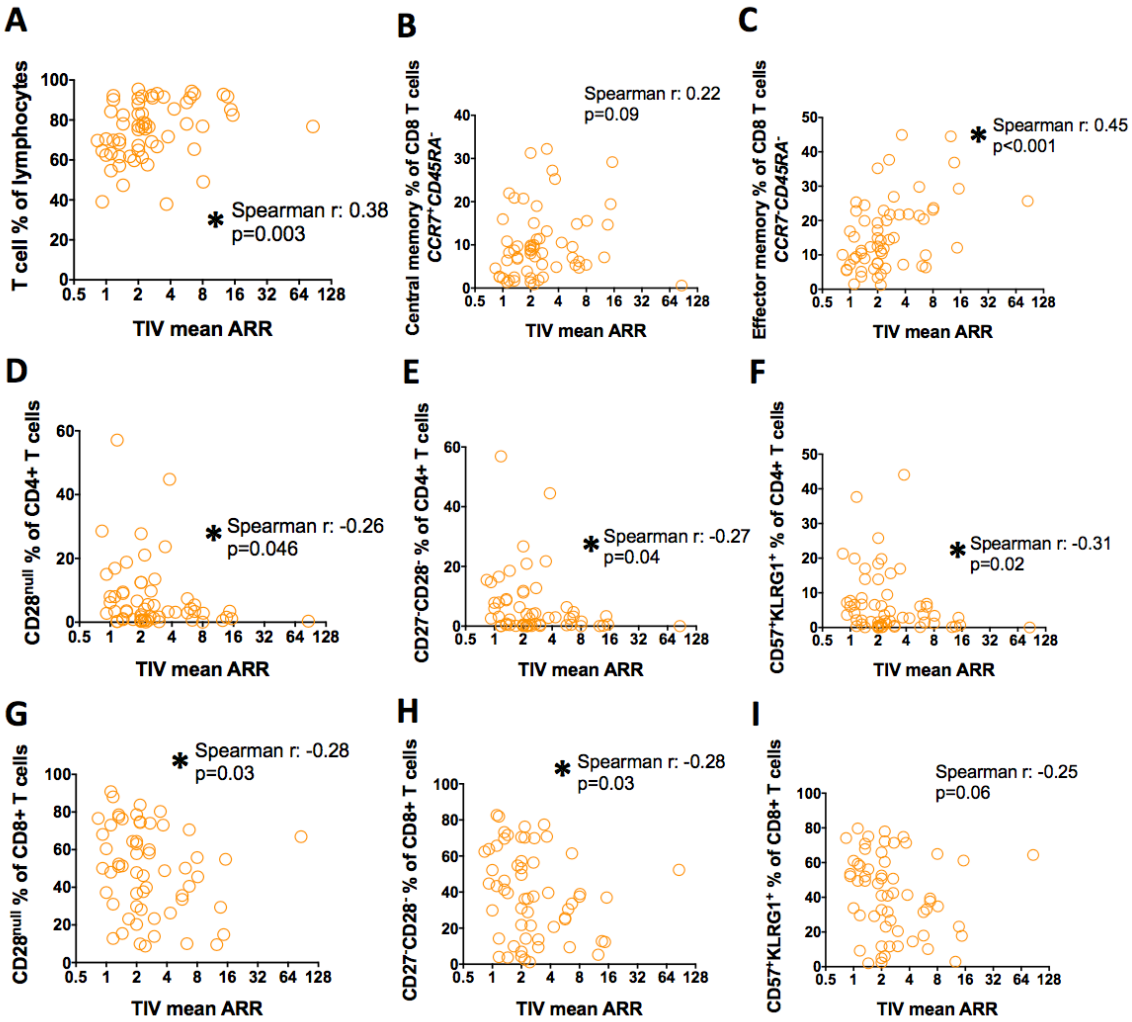
In contrast to the above, and despite the strong association between latent CMV infection and PPV23 vaccine response, there were no significant correlations between any of the T cell "senescence"-associated phenotypes described earlier in this Chapter and PPV23 mean ARR (Table 5-3). This may be a reflection of the T-independent nature of the antigens contained in the vaccine (i.e. that a difference truly does not exist) or due to the small sample size in this study.

When naïve and memory T cell phenotypes were assessed using the surface markers CCR7 and CD45RA, expansions of the effector memory ($CCR7^{-}CD45RA^{-}$) population of $CD8^{+}$ T cells were significantly related to higher TIV ARR (Figure 5-22 B). Similarly, expansions of central memory ($CCR7^{+}CD45RA^{-}$) $CD8^{+}$ T cells approached significance (Figure 5-22 C). This could suggest that the humoral response to TIV observed in this study is supported by memory T cell responses, for example, directed against conserved influenza antigens, which afford a degree of cross-reactivity between different influenza strains e.g. nucleoprotein and matrix protein 1 (described in Chapter 1, Introduction). Indeed, it is highly likely that older adults would have established a sizeable pool of influenza-antigen memory lymphocytes from repeated exposure to multiple circulating influenza strains over their lifetime.

No relationships were observed between naïve and memory populations of $CD4^{+}$ or $CD8^{+}$ T cells and PPV23 mean ARR, which is in keeping with the T-independent nature of antigens in this vaccine.

Figure 5-22 Relationships between T cell phenotypes and TIV response.

Relationships shown between TIV mean ARR and T cell % (A); $CD8^+$ T cell central memory (B) and effector memory % (C); $CD4^+$ T cell $CD28^{null}$ (D) $CD27^-CD28^-$ (E) and $CD57^+KLRG1^+$ % (F) and $CD8^+$ T cell $CD28^{null}$ (G) $CD27^-CD28^-$ (H) and $CD57^+KLRG1^+$ % (I). Non-parametric correlation statistics shown. P value <0.05 considered significant – denoted by *.

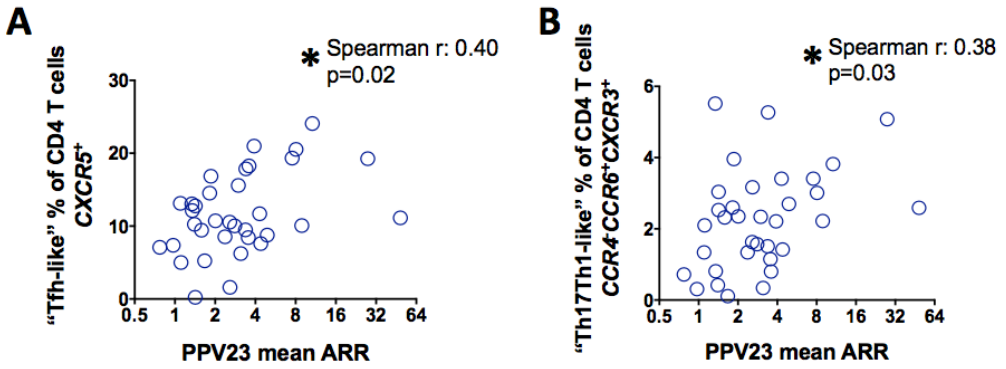


No significant relationships were seen between TIV mean ARR and proportions of $CD4^+$ and $CD8^+$ T cells (or CD4/8 ratio), or expansions of different helper and regulatory T cell

phenotypes in the $CD4^+$ T cell compartment (Table 5-3). However, greater proportions of circulating "Tfh-like" ($CXCR5^+$) and "Th1Th17-like" ($CCR4^-CCR6^+CXCR3^+$) $CD4^+$ T cells were significantly associated with higher PPV23 mean ARR (Table 5-3 and Figure 5-23). This is unexpected, as TI responses classically do not engage Tfh cells as in TD germinal centre reactions. The secretory phenotype of $CCR4^-CCR6^+CXCR3^+$ ("Th1Th17-like") T cells has previously been described as dominated by IFN-γ, but also includes IL-17 (15). Co-administration of IFN-γ has previously been shown to improve murine immune responses, albeit to TD antigens (306), but it has also been shown to suppress APC function (307). The importance of IL-17 in driving antibody production is debated (308). As such, the associations between PPV23 mean ARR and expansions of "Tfh-like" and "Th1Th17-like" $CD4^+$ T cells seen in this study require corroboration in larger studies of patients with CKD and also further interrogation as to their true functional phenotype.

Figure 5-23 Relationships between PPV23 mean ARR and $CD4^+$ T cell proportions of "Tfh-like" (A) and "Th1Th17-like" (B) cells.

Non-parametric correlation statistics shown. P value <0.05 considered significant – denoted by *.



5.7 Discussion

Robust antigen-specific T cell responses are fundamental for the clearance of pathogens and long-term health of the host. Given the increased susceptibility to infection seen in patients with CKD in both this study and previous literature (146, 149), together with previously reported impairments in responses to TD vaccine antigens (154, 179), I expected to see a difference in T cell phenotype between the two groups in this study. As such, it was surprising that the differences observed were minimal and that the main differences in T cell phenotypes observed within the study cohort were dependent on CMV serostatus.

In this study, older patients with CKD demonstrated a T lymphopenia compared to age matched healthy controls, but with a preserved CD4/8 ratio, contrary to previous literature (214). I observed no contraction of naïve $CD4^+$ or $CD8^+$ T cell populations in patients with CKD (contrary to previous reports in patients with ESRD (186, 214)), and only an expansion of central memory $CD8^+$ T cells, which appeared to be CMV-independent. T_{EMRA} proportions of both $CD4^+$ and $CD8^+$ T cell compartments were also equivalent in patients with CKD in this study, compared to healthy age matched individuals, also contrary to previous literature on T cells in patients with ESRD (186). No differences in the size of the "senescence"-associated T cell populations ($CD28^{null}$, $CD27^-CD28^-$, $CD57^+KLRG1^+$ or $CD28^-CD57^+$ proportions of $CD4^+$ or $CD8^+$ T cells) were seen between patients with CKD and controls in this study, again, contrary to previous reports (186, 215, 218).

There are several possible explanations as to why the findings of this study do not agree with previous literature. In this study, patients with CKD were well-matched for age and

CMV seropositive individuals had equivalent CMV-specific IgG titres – considered a surrogate for the immune "imprint" of CMV (95). As such, a strength of this study is the inherent controlling for the effects of age and latent CMV infection on the immune system as potential confounders. Previous studies in CKD/ESRD populations generally report the presence of latent CMV only as a binary variable (seropositive/seronegative), without consideration of the magnitude of the CMV-specific humoral response. Therefore, differences in immune cell profiles (particularly T cells), may have previously been ascribed to CKD, when they may, in reality, reflect differences in CMV-specific responses. This requires further interrogation in a larger cohort of patients with CKD and age-matched controls. Interestingly, when all study participants were taken into account, poor humoral responses to a T-dependent vaccine – TIV, were significantly associated with expansions of T cell populations associated with latent CMV infection in this study. This suggests that latent CMV infection may play a significant role in modulating responses to T-dependent antigens in older adults with and without chronic disease – a feature that has previously been observed by others (96, 309).

Another possible explanation is that dialysis therapy (excluded in this study), rather than renal impairment, could be driving the T cell features previously described in patients with ESRD. As the sample size of this study is small, it is possible that I have not been able to see a difference when it actually exists. However, the strong relationships between latent CMV infection and "senescence"-associated and naïve/memory T cell populations in this study suggest this is less likely.

Another important finding of this study is the expansion of "Th2-like" and T_{reg} proportions of $CD4^+$ T cells in patients with CKD, compared to age matched controls, which appears to be independent of latent CMV infection. Again, the majority of previous literature

suggests the presence of a Th1 skew in CKD/ESRD (302), with reductions in circulating regulatory T cell numbers and function (230). However, several studies have recently shown a similar $CD4^+$ helper phenotype in patients with CKD/ESRD as seen here (226, 227, 310), which could be related to vitamin D deficiency (310). I did not evaluate serum vitamin D levels in this study and therefore this could be a confounder here.

Previous studies have largely used lineage-specific transcription factors and cytokine secretion profiles to define helper $CD4^+$ T cell populations in patients with CKD/ESRD. The use of surface chemokine receptor profiling in this study may, in part, account for the different findings in this study. I did not perform any functional T cell assays to examine the cytokine profiles of the helper T cell populations defined by surface chemokine receptors, hence I cannot be totally sure that the "Th2-like" population are of the Th2 functional phenotype.

Interestingly, latent CMV infection has previously been shown to induce a Th1 dominant $CD4^+$ T cell effector profile (311-313). Indeed, in this small study, there was a slight increase in the proportion of "Th1-like" $CD4^+$ T cells in CMV seropositive individuals, compared to those that were seronegative, and a positive association between "Th1-like" expansions and the size of "senescence"- and CMV-associated populations. As such, previous literature demonstrating this in CKD may have been confounded by the effect of CMV on the immune system. This requires further interrogation in a larger cohort of patients with CKD and age-matched controls.

Chronological ageing is associated with Th2 polarisation of the $CD4^+$ T cell compartment in both humans (314) and mice (315), together with expansion of T_{reg} populations (316). As such, the features associated with CKD in this study may well reflect the previously

postulated “accelerated immune ageing” process (142) that may be independent of CMV. Immune “ageing” and the development of proliferative senescence occurs as a result of a number of processes, which include telomere shortening, accumulation of DNA damage and proliferation in response to chronic antigen exposure (e.g. CMV) (79). Previous studies have proposed that immune dysfunction associated with CKD is related to the action of retained uraemic toxins (182). Although previous studies (317, 318) have shown telomere shortening in patients with ESRD compared to age-matched controls, the significant effect of CMV on telomere attrition (319) has not been accounted for. As such, I suggest that features of accelerated ageing in CKD, as seen in this study, could be driven by accelerated DNA damage and/or impairment of DNA repair mechanisms driven by the uraemic milieu, supporting evidence for which already exists in the literature (320, 321).

As with previous results, multiple comparisons have been made between data from patients with CKD and controls in this chapter. Although some of the significant findings may represent type I errors (false positives), most are probably due to genuine differences between the disease groups and associations between variables.

CHAPTER 6

B LYMPHOCYTES IN OLDER ADULTS WITH CKD

6.1 Introduction

Upon activation with cognate antigen, B lymphocytes differentiate into antibody secreting cells (ASCs) and antigen-specific memory B cells. This process usually occurs as a result of interactions with T cells also specific for the antigen (T-dependent), but can also occur independently of T cell help. In this study of older individuals with CKD I have used two vaccines as an "in vivo" antigen challenge – one that is T-dependent (TIV) and one that is T-independent (PPV23), and have evaluated the humoral response to both vaccines in Chapter 4 (Vaccine response). As such, I thought it pertinent to also examine the composition of the B cell compartment alongside analysis of vaccine-specific antibody.

As described in Chapter 1 (Introduction), previous studies have reported various B cell phenotypic and functional changes associated with severe CKD and/or dialysis treatment, including a generalised B lymphopenia, contraction of naïve B cell population and increased B cell apoptosis (232, 233, 235, 236). These features are similar to what has been described in association with immune ageing, suggesting the presence of an accelerated "ageing" process in the B cell compartment in renal disease. At present it is not clear whether the same features are present in pre-dialysis non-immune CKD.

This Chapter presents results of B lymphocyte phenotyping performed on patients with CKD and age-matched controls. As with the analysis of T cell phenotype presented in Chapter 5 (T cells), samples from 2 individuals were also excluded from this analysis due to abnormally high B cell proportions of total lymphocytes – a feature consistent with the premalignant condition monoclonal B-cell lymphocytosis (previously reported to have tumour-associated genetic mutations (295)). Cross-sectional phenotyping was performed on PBMC samples with a minimum viability of 80% collected either at the baseline study

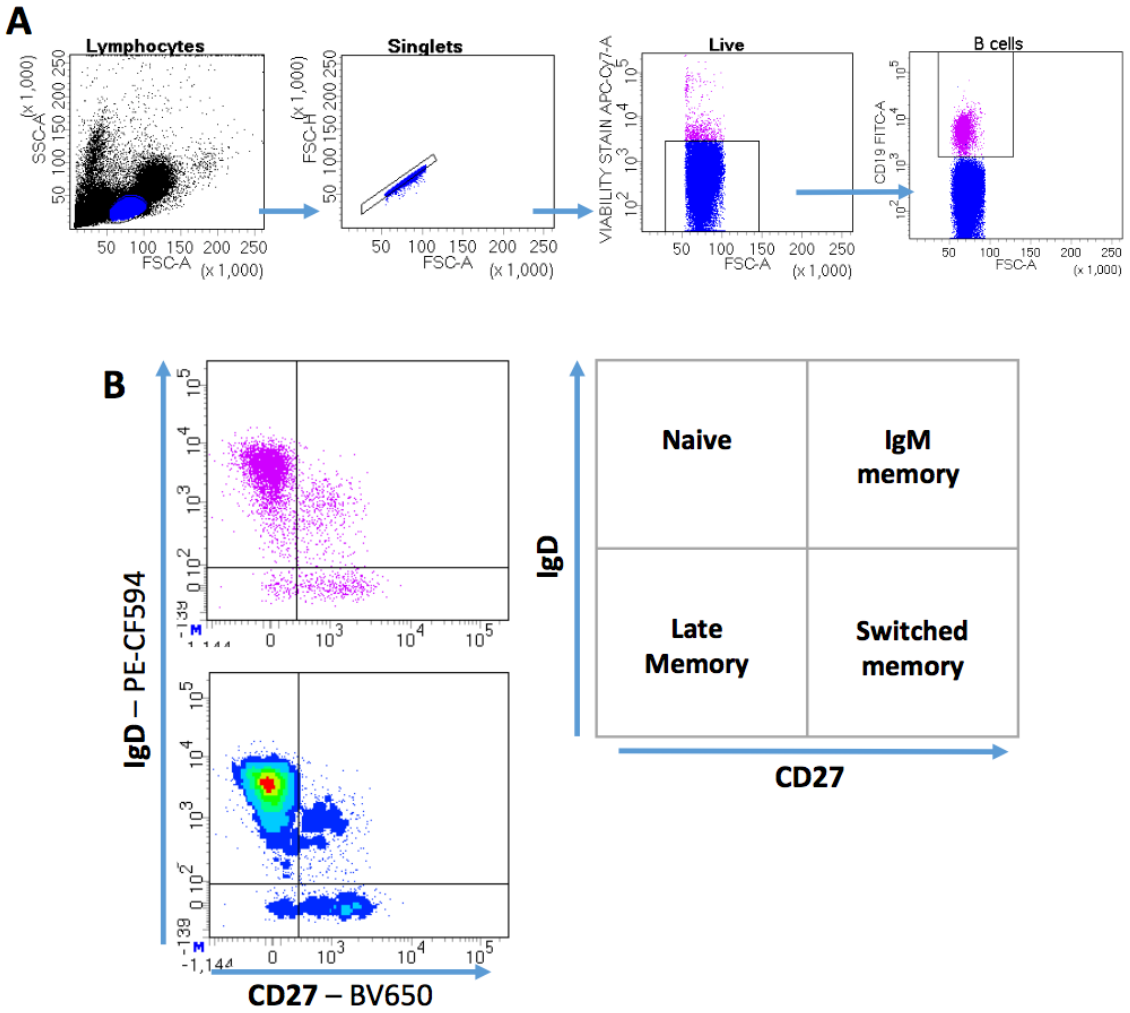
visit (pre-vaccination) or at month 6 post-vaccination. The final sample size of the cross-sectional B cell phenotype analysis: 28 patients with CKD and 27 controls, was determined by practical constraints including assay failure and viability of stored samples. In this sample, a significantly greater proportion of patients with CKD were CMV-seropositive than controls (n=24 versus 15, Fisher's exact p=0.03). Although effects of latent CMV infection on the B cell compartment remain incompletely understood (93, 94, 322), given the significant effects of CMV on the T cell phenotypes described in Chapter 5 (T cells), CMV serostatus is included in the following analysis of B cells as a potential confounder, with unexpected results.

Dynamic changes in circulating plasma cell/blast populations from pre-vaccination to day 7 post-vaccination were also assessed in a subset of study participants as a surrogate for antigen-specific B cell responses (10 patients with CKD, 7 age-matched controls). To date, no studies have been performed in non-dialysis CKD patients to evaluate this, so our results represent a novel insight into this aspect of B cell function in renal impairment.

6.2 Multimorbidity is associated with B lymphopenia in older adults, but preserved naïve/memory B cell proportions

Circulating B lymphocytes were identified from frozen PBMCs by surface expression of $CD19^+$ (Figure 6-1A) and further subcategorised into naïve and memory subtypes using the markers CD27 and IgD (Figure 6-1B) as per previously published methods (323, 324).

Figure 6-1 Gating strategy for identification of B cells and naïve/memory B cell subtypes. Singlet live lymphocytes were identified by forward/side scatter characteristics and viability dye (APC-Cy7) – scatter plots shown. B cells were defined by surface expression of CD19 (A) and naïve/memory subtypes by surface expression of IgD and CD27 (B – representative scatter plot shown in top panel, density plot shown in bottom panel).

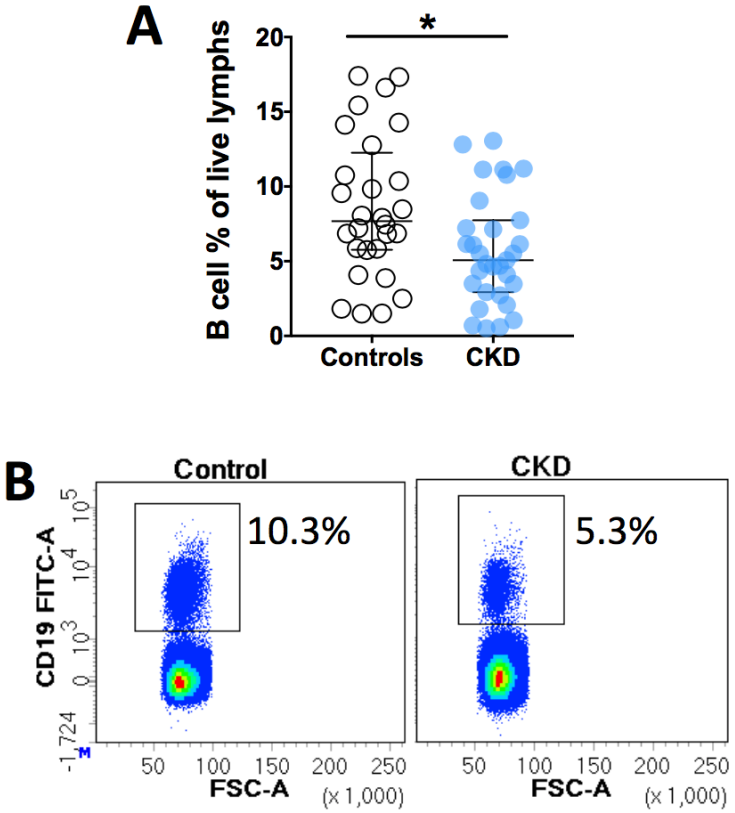


B cell proportion of total lymphocytes was significantly reduced in patients with CKD compared to controls (Figure 6-2). Contraction of the B lymphocyte pool was associated with reducing eGFR (Pearson's correlation coefficient 0.31, 2-tailed p=0.02 for both

MDRD and CKD-EPI eGFR), but not degree of proteinuria (ACR), glycaemic control (HbA1c) or inflammation (hsCRP). However, B cell proportion of total lymphocytes was also inversely related to both CCI and medication burden (Spearman correlation coefficient -0.37, 2-tailed p=0.004 and Spearman correlation coefficient -0.42, 2-tailed p=0.001, respectively) and to CMV-specific IgG titre (Pearson R= -0.28, 2-tailed p=0.03).

Figure 6-2 B lymphocytes in SONIC study participants.

B cell proportions (A) in patients with CKD and controls. Representative flow density plots from one control and one patient with CKD shown in panel B. Error bars show median and IQR. * denotes p<0.05.



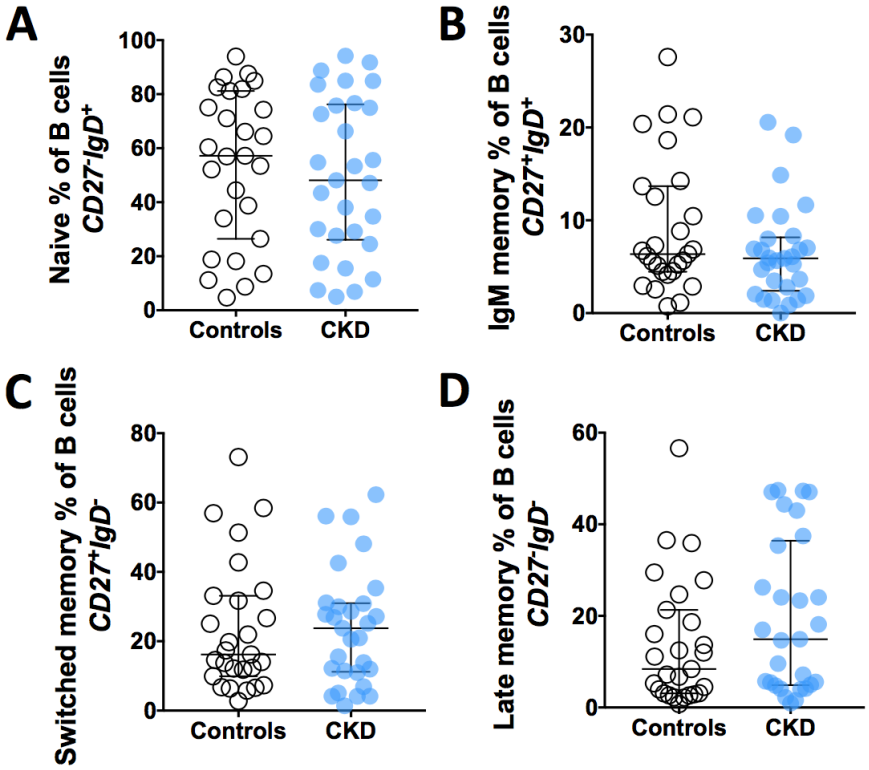
CCI, medication burden (both measures of multimorbidity) and CKD status were significantly collinear and, therefore, their independent contribution to a multivariate predictive model could not be reliably determined. They were each, however, in turn, significant predictors of B cell proportion of total lymphocytes in a linear regression model that also included age, gender and CMV serostatus. The model that included medication burden best explained the variability in B cell proportion of total lymphocytes, compared to CCI or CKD status (adjusted R^2=0.23 versus 0.17 and 0.14, respectively). No additional improvement in this predictive model was seen with the subsequent addition of CKD (R^2 did not increase), suggesting that, in this study, the presence or absence of renal impairment had no extra predictive effect on B cell proportion in addition to medication burden as a measure of multimorbidity.

No significant differences were seen in the proportion of naïve/memory B cells between patients with CKD and controls (Figure 6-3).

A greater degree of B lymphopenia was significantly associated with smaller naïve proportions (Pearson R: 0.29, p=0.03) and larger late memory proportions (Pearson R: -0.33, p=0.01) of total B cells. This suggests that, in individuals from this study, as the proportion of circulating B cells declines, there is a shift within the B cell population towards a memory phenotype - features consistent with previously reported B cell changes associated with chronological ageing (83).

Figure 6-3 Naive/memory B cell phenotype assessment in patients with CKD and controls, based on CD27 and IgD expression.

Naïve/memory B cell phenotypes shown as proportions of total B cells . Error bars show median and IQR.



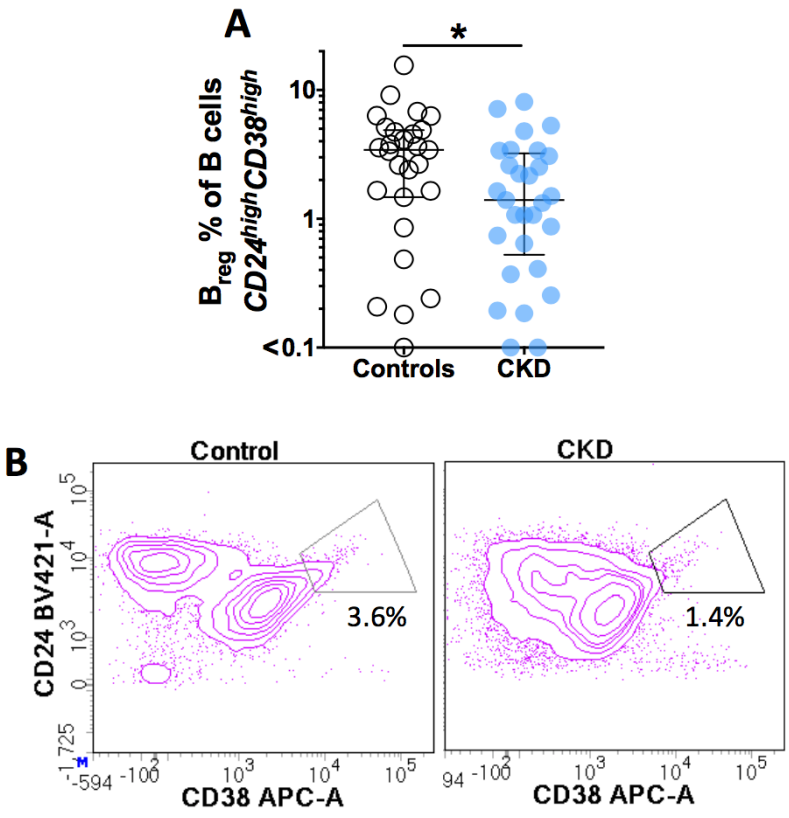
No significant associations were observed between naïve or memory B cell proportions in this study and eGFR (MDRD or CKD-EPI), ACR, HbA1c, CCI or medication burden, but the proportion of IgM memory B cells ($CD27^+IgD^+$) did inversely correlate with age (Pearson's coefficient -0.36, 2-tailed p=0.006), CMV-specific IgG titre (Pearson's coefficient -0.27, p=0.046) and hsCRP (Pearson's coefficient -0.32, p=0.02). Indeed, linear regression analysis revealed both age and CMV serostatus to be significant predictors of IgM memory % of B cells (p=0.03 for both variables), independent of gender and CKD status, and when the collinear variables CCI or medication burden were substituted for CKD status in this model.

6.3 Multimorbidity is associated with a contraction of the B_{reg} pool in older adults

Regulatory B cells (B_{regs}) were defined by surface markers as $CD19^{+}CD24^{high}CD38^{high}$ as per previously published methods (85, 325) – gating shown in Figure 6-4 A. B_{reg} proportions of total B cells were significantly reduced in patients with CKD compared to controls (Figure 6-4 B, C).

Figure 6-4 Evaluation of B_{reg} populations in patients with CKD and controls.

Comparisons between controls and patients with CKD of B_{reg} proportions of total B cells (A). Panel B shows representative flow contour plots for patients with CKD and controls – B_{regs} defined as $CD19^{+}CD24^{high}CD38^{high}$. Error bars show median and IQR. *denotes Mann-Whitney 2-tailed $p<0.05$.



A smaller B_{reg} proportion of total B cells was significantly associated with smaller naïve (Pearson R: 0.71, p<0.0001) and larger late memory proportions of total B cells (Pearson R: -0.86, p<0.0001), suggesting that contraction of the B_{reg} pool is associated with a skew towards a B cell memory phenotype. This is consistent with the previously reported age-associated decline in both naïve and B_{reg} populations seen in healthy older adults (85).

Lower B_{reg} proportions of total B cells were significantly associated with elevated CCI (Spearman correlation coefficient -0.35, p=0.009), but not with medication burden, ACR, HbA1c, hsCRP or CMV-specific IgG titre. Declining eGFR was generally associated with lower B_{reg} proportions of B cells, but this fell just short of statistical significance (Pearson's correlation coefficient 0.26, 2-tailed p=0.052 for MDRD and 0.25, p=0.07 for CKD-EPI eGFR).

CCI was a significant independent predictor of B_{reg} proportion of total B cells in a linear regression model that also included age, gender and CMV status (p=0.04). The addition of the collinear variables CKD status or eGFR did not improve model fit (R^2 unchanged). This suggests that, in this study, renal impairment does not appear to have an extra effect on B_{reg} populations above that of CCI – a more comprehensive measure of multimorbidity.

6.4 Renal impairment and magnitude of humoral response to latent CMV infection are associated with upregulation of B cell activation markers CD80/86, but not CD40

Ligation of the B cell surface costimulatory molecule CD40 by its Tfh-derived ligand CD40L stimulates class switch recombination in activated B cells (3), leading to the

production of a robust long-lasting antibody response. B cells also express surface CD80 and CD86, which act as ligands for the T cell co-stimulatory receptor CD28, thus playing a key role in their function as APCs. Having already examined the humoral response to vaccination in this study, together with naïve and memory B cell populations, I then went on to evaluate surface expression of CD40, CD80 and CD86 on B cells, as a surrogate for their capacity to interact with cognate T cells after activation. As both CD80 and CD86 are ligands for the T cell receptor of interest (CD28), I chose to use the same fluorochrome (PE-Cy7) for both, which enabled me to also interrogate the naïve/memory B cell populations in the same flow cytometry panel (see Chapter 2, Methods). In the rest of this analysis, I will therefore refer to expression of these surface markers as CD80/86 as it is not possible to distinguish between them in design of the flow cytometry panel. Representative gating and controls for this analysis are shown in Figure 6-5.

Patients with CKD had equivalent proportions of B cells expressing CD40 compared to controls, but significantly higher proportions expressing CD80/86, which translated into greater proportions of B cells expressing all of these markers ($CD40^{+}CD80/86^{+}$) – shown in Figure 6-6.

Expansions of $CD80/86^{+}$ populations of B cells were significantly associated with reduced B cell proportion of total lymphocytes, contraction of naïve and regulatory proportions of B cells and expansions of switched and late memory proportions (Figure 6-7) – features previously reported in association with an “ageing” immune system (83, 85).

Figure 6-5 Representative gating for identification of CD40 and CD80/86 expression on B cells.

Lymphocytes were first identified by forward and side scatter characteristics, then gated on singlets and live cells (as shown previously). CD19+ cells were then examined for expression of both CD40 and CD80/86. FMO controls were initially used to define gate position, but this was very closely approximated by gating on CD19- lymphocytes (mainly T cells). Hence, this "internal control" was used in subsequent analyses and an example is shown in panel A (scatter plots shown). Panel B shows representative results from controls and patients with CKD, percentages shown denote double positive (CD40+CD80/86+) proportion of total B cells (contour plots shown).

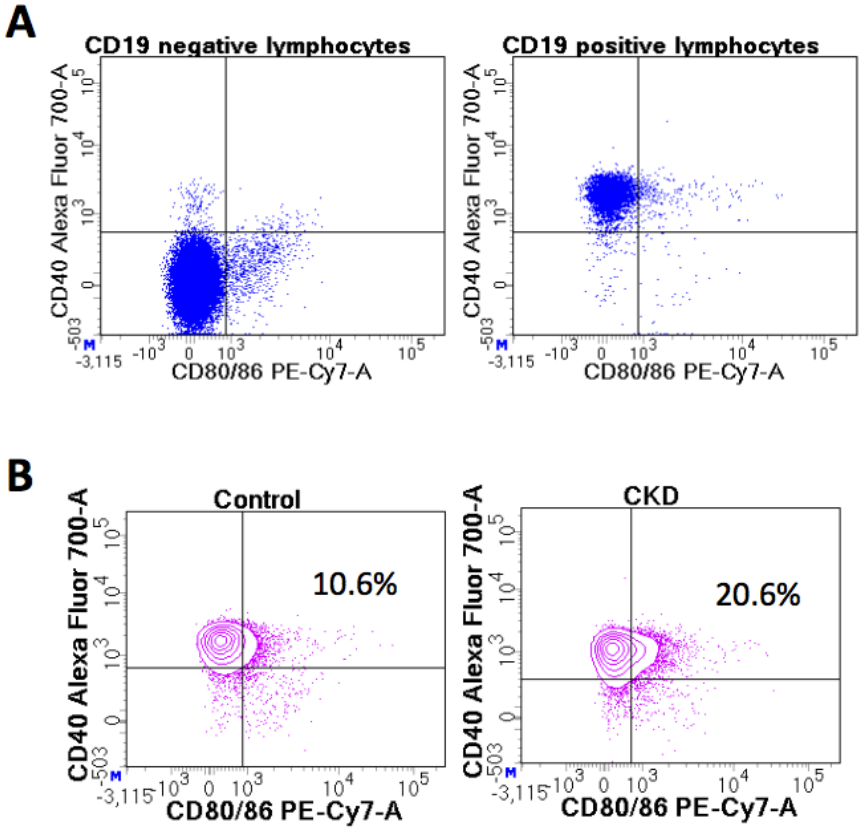


Figure 6-6 Expression of co-stimulation markers CD40 and CD80/86 on B cells from patients with CKD and controls.

Proportions of B cells expressing surface CD40 (A), CD80/86 (B) or both (C). *denotes unpaired t-test 2-tailed $p<0.05$. Error bars show median and IQR.

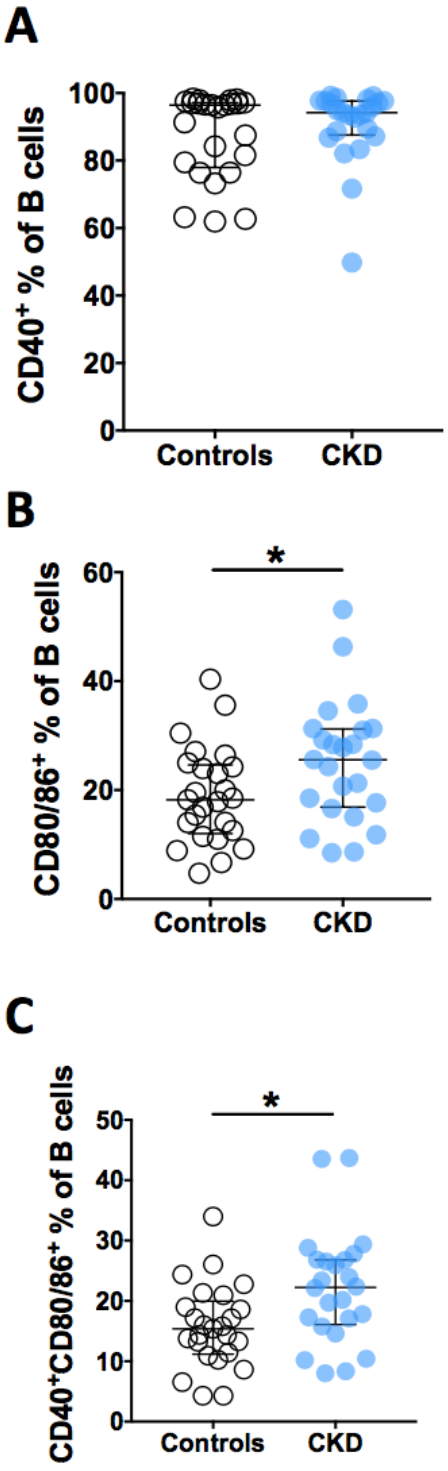
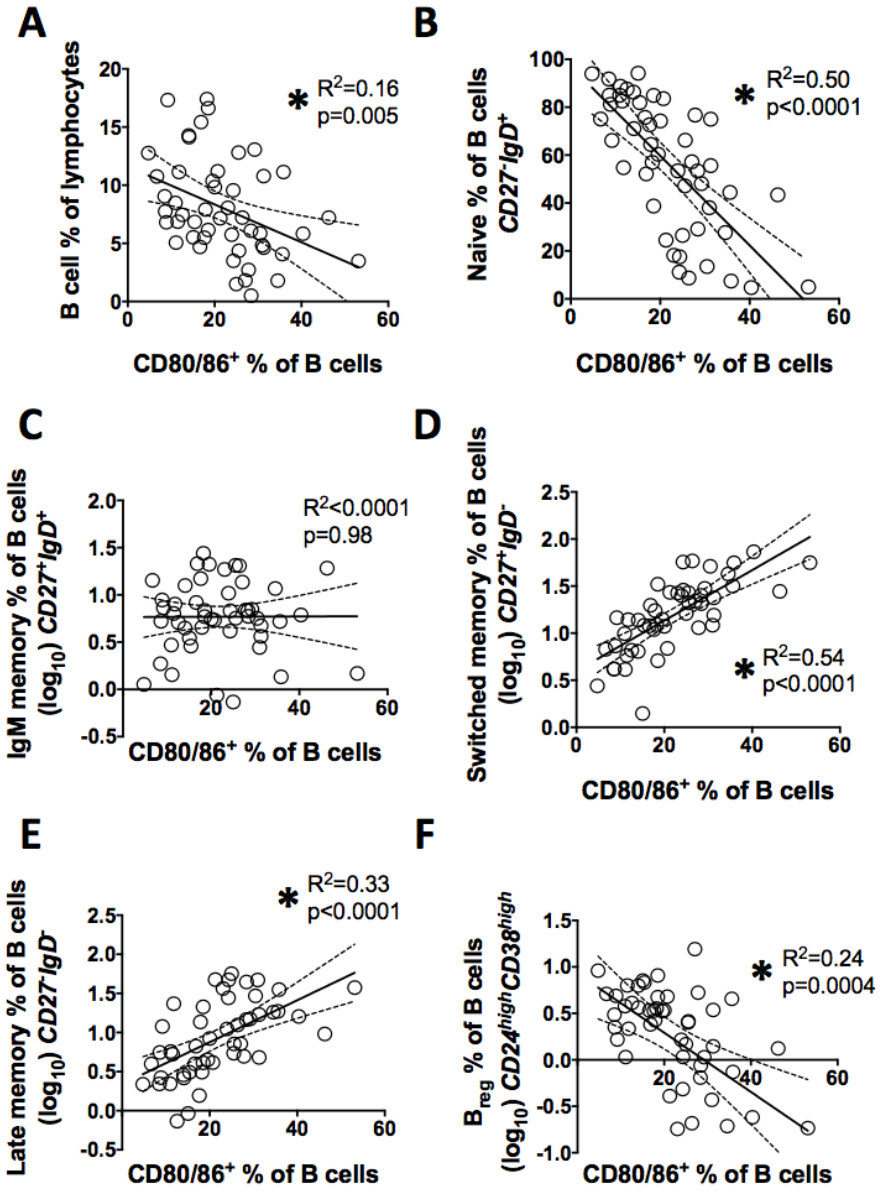


Figure 6-7 Relationships between CD80/86^{+} B cell proportions and other B cell subsets.

Correlation shown between CD80/86^{+} B cell proportions and total B cell % (A), proportions of naïve (B), IgM memory (C), switched memory (D), late memory (E) and regulatory B cells (F). Linear regression statistics shown and best fit line plotted with 95% CI, p value<0.05 deemed significant and denoted by *.



I then went on to investigate whether the observed increase in the proportion of B cells expressing CD80/86 could be accounted for by a subtle skew towards a B cell memory phenotype in patients with CKD. Indeed, when all study participants were considered, the proportion of B cells expressing CD40 reduced with loss of IgD, whereas CD80/86 expression followed the opposite pattern, with significantly increased proportions of antigen-experienced B cells expressing these markers than naïve cells (Figure 6-8 A, B). A significant inverse correlation was also observed here between proportions of B cells expressing CD40 and CD80/86 (Figure 6-8 C). Patients with CKD had significantly higher proportions of B cells expressing CD80/86 than controls in IgM memory and late memory subtypes and this approached significance for naïve B cells (Mann-Whitney 2-tailed p=0.1) - Figure 6-8 E. This suggests the presence of a disease effect on CD80/86 expression that appears to be independent of antigen experience. When expression of both CD40 and CD80/86 was considered, patients with CKD had higher proportions of all naïve/memory populations expressing these markers than controls (Figure 6-8 F).

Measures of renal function (MDRD or CKD-EPI eGFR), but not kidney damage (baseline ACR) significantly correlated with CD80/86^{+} proportion of B cells (Figure 6-9). Interestingly, no significant associations were seen between CD80/86^{+} B cell proportion and measures of multimorbidity (CCI, medication burden), systemic inflammation (hsCRP or NLR) or glycaemic control (HbA1c). These relationships suggest that the degree of renal impairment, rather than systemic inflammation or the presence of multimorbidity, had a greater impact on expansions of CD80/86^{+} populations in the B cell compartment in this study of older adults with and without chronic disease.

Figure 6-8 Expression of CD40 and CD80/86 on B cell populations depending on antigen experience.

Results shown from all study participants in A, B, C; comparisons between controls and patients with CKD shown in: D-F. $CD40^+$ B cell populations by naïve/memory subtypes shown in A and D. $CD80/86^+$ B cell populations by naïve/memory subtypes shown in B and E. Panel C shown linear regression statistics and best fit line (with 95% CI) of relationship between $CD40^+$ and $CD80/86^+$ proportions of total B cells in all study participants. Panel F shows the distribution of $CD40^+CD80/86^+$ B cells by naïve/memory subtypes compared by disease group. Error bars show median and IQR unless stated. *denotes Mann-Whitney 2-tailed $p<0.05$.

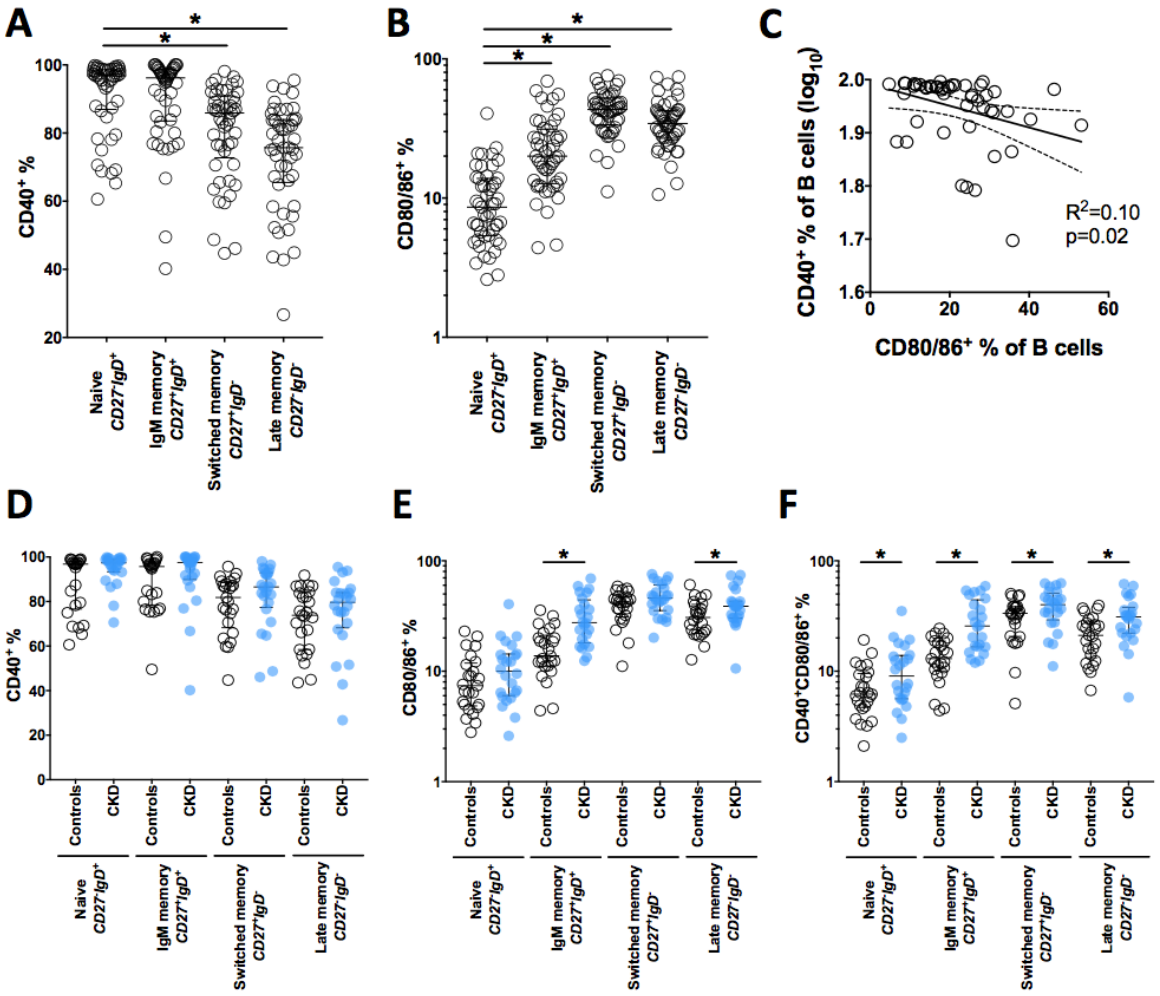
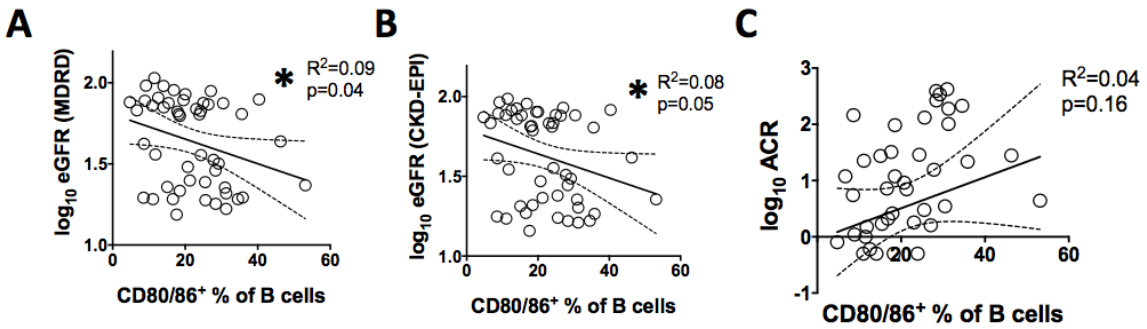


Figure 6-9 Relationships between CD80/86$^+$ proportions of B cells and markers of renal disease.

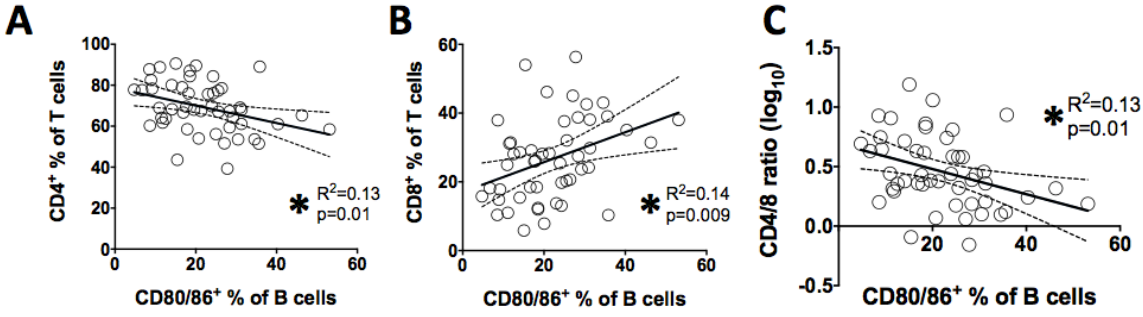
Linear regression statistics and best fit line (with 95% CI) shown for relationships between CD80/86$^+$ proportions of B cells and A: MDRD eGFR, B: CKD-EPI eGFR, and C: baseline ACR. P value <0.05 considered significant and denoted by *.



Contraction of CD4$^+$ and expansion of CD8$^+$ proportions of T cells (together with reductions in the CD4/8 ratio) were significantly associated with expansions of CD80/86$^+$ B cells in both controls and patients with CKD (Figure 6-10).

Figure 6-10 Relationships between CD80/86$^+$ B cell proportions and basic T cell subsets.

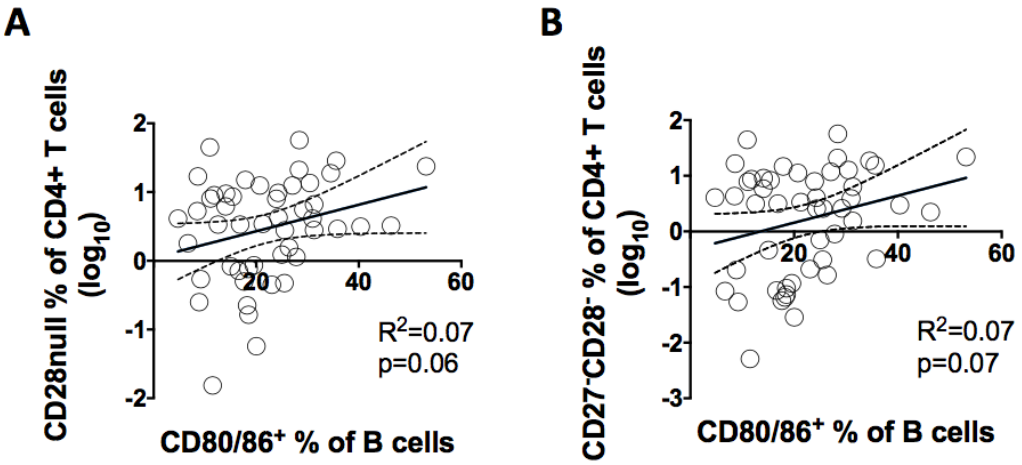
Linear regression statistics and best fit line (with 95% CI) shown for relationships between CD80/86$^+$ proportions of B cells and CD4$^+$ (A) and CD8$^+$ (B) proportions of total T cells, together with their ratio (C). *denotes statistical significance (p<0.05).



Given the integral role of CD80/86 interaction with T cell CD28, I then examined the relationships between this B cell population and T cell phenotypes lacking surface expression of CD28, as described in Chapter 5 (T cells). Positive correlations between $CD80/86^{+}$ proportions of B cells and expansions of $CD28^{null}$ and $CD27^{-}CD28^{-}$ populations of $CD4^{+}$ T cells fell just short of significance (Figure 6-11), but no significant relationships were seen with $CD28^{null}$ or $CD27^{-}CD28^{-}$ proportions of CD8 T cells. This suggests that the observed expansion of $CD80/86^{+}$ populations of B cells seen in patients with CKD in this study may be compensatory to downregulation of their ligand CD28 on CD4 T cells.

Figure 6-11 Relationships between $CD80/86^{+}$ B cell proportions and $CD28^{null}$ (A) and $CD27^{-}CD28^{-}$ (B) $CD4^{+}$ T cell populations.

Linear regression statistics and best fit line (with 95% CI) shown. P value <0.05 considered significant.



As the loss of surface expression of CD28 on CD4 T cells is strongly associated with latent CMV infection (91, 92, 326), I then investigated the relationship between the size of CD80/86^{+} B cell populations and presence of latent CMV infection in this study. CMV seropositive patients with CKD tended to have greater proportions of CD80/86^{+} B cells than seronegative individuals (statistical analysis limited by sample size, n=3 seronegative patients with CKD) and also compared to CMV seropositive controls (Figure 6-12 A). This may suggest a different CMV-associated effect on this B cell population in the presence of renal impairment, compared to healthy individuals.

However, I observed significant positive correlations between CMV-specific IgG titre and CD80/86^{+} % of B cells when CMV seropositive controls and patients with CKD were considered together (Figure 6-12 B) or separately (Figure 6-12 B, C). As such, the magnitude of the humoral CMV-specific response appears to be associated with CD80/86^{+} B cell expansions in older adults with and without chronic disease.

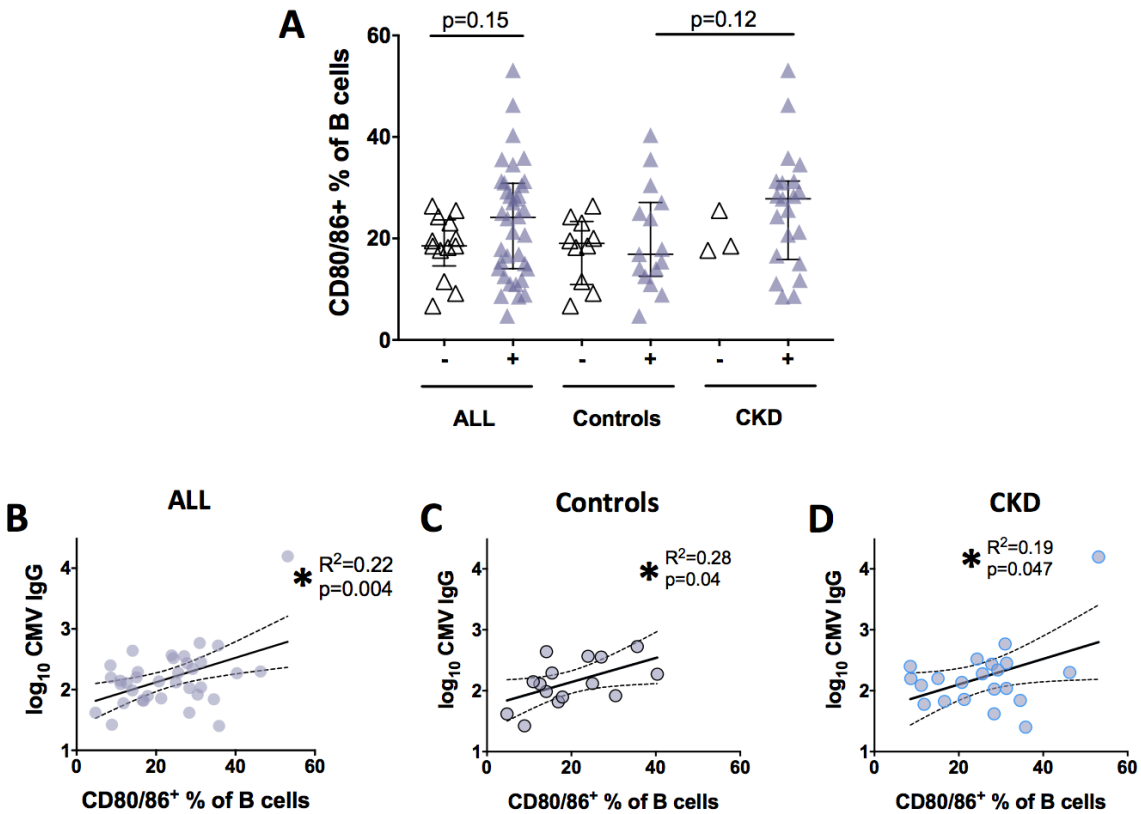
CKD status was the only significant predictor of CD80/86^{+} B cell % in a linear regression model that also included age, gender and CMV-specific IgG titre ($p=0.047$). When CKD status was substituted by CCI or medication burden, none of the variables in the model approached significance at the 0.05 level, which suggests that the effect of renal impairment is greater than that of concomitant multimorbidity. However, when CMV seropositive individuals were evaluated separately, CMV-specific IgG was the only significant predictor of CD80/86^{+} B cell expansions independent of age, gender and CKD status ($p=0.03$).

These findings suggest that both renal impairment and the magnitude of the humoral immune response to latent CMV infection (resulting in expansions of CD4 T cell

populations lacking the costimulatory receptor CD28) could have an effect on expanding populations of CD80/86^{+} B cells in older adults. This appears to be independent of the degree of systemic inflammation (hsCRP) and multimorbidity/polypharmacy in this study.

Figure 6-12 Relationship between CD80/86 expression on B cells and latent CMV infection.

CD80/86+ proportions of total B cell proportion compared by CMV serostatus (+/-) and disease group are shown in panel A (unpaired t test 2-tailed p values shown). The relationship between CD80/86^{+} proportion of B cells and CMV-specific IgG titre is shown in B for all CMV-seropositive participants, in C for only CMV-seropositive controls and in D for only CMV-seropositive patients with CKD (linear regression statistics and best fit line with 95% CI shown). Error bars show median +/- IQR unless stated. P value <0.05 considered significant and denoted by *.



6.5 Generation of circulating plasma blasts/cells at day 7 post-vaccination is maintained in patients with CKD

A sample of study participants were assessed for generation of circulating ASCs at day 7 (10 patients with CKD and 7 age-matched controls). ASCs were defined as $CD19^{+}CD20^{-}CD38^{high}$ as per previously published methods (327, 328) with representative gating shown in Figure 6-13 A, B. Unfortunately, the detection of the surface proteoglycan CD138 (the hallmark of mature plasma cells (329), differentiating them from plasmablasts) was very variable after cryopreservation of PBMCs in this study (infact, completely abrogated in some post-vaccination samples during assay development as shown in Figure 6-13 C), thus limiting its utility in flow cytometry analysis of frozen samples here. As such, the $CD19^{+}CD20^{-}CD38^{high}$ ASC population described in this analysis will, henceforth, be referred to as plasma blasts/cells (PB/PC) as I was not able to confidently determine CD138 expression in frozen samples.

Both patients with CKD and controls were able to expand circulating PB/PC populations at day 7 post-vaccination, approximately to the same degree (Figure 6-14).

Figure 6-13 Representative plasma blast/cell gating strategy.

Lymphocytes were first identified by forward and side scatter characteristics, then gated on singlets and live cells (as shown previously). $CD19^{+}$ cells were then examined for surface expression of CD20 and CD38, with plasma blasts/cells defined as $CD20^{-}CD38^{high}$. Panels A and B show representative flow scatter plots for samples from controls and patients with CKD, respectively. Panel C shows abrogation of CD138 staining in PBMC samples used in assay development: cells from a healthy laboratory donor were taken at day 7 after TIV received as part of routine care for that individual; cells were stained immediately after isolation (left scatter plot) and an aliquot was frozen at -80˚C and analysed at a later stage with the same flow cytometry technique (right scatter plot).

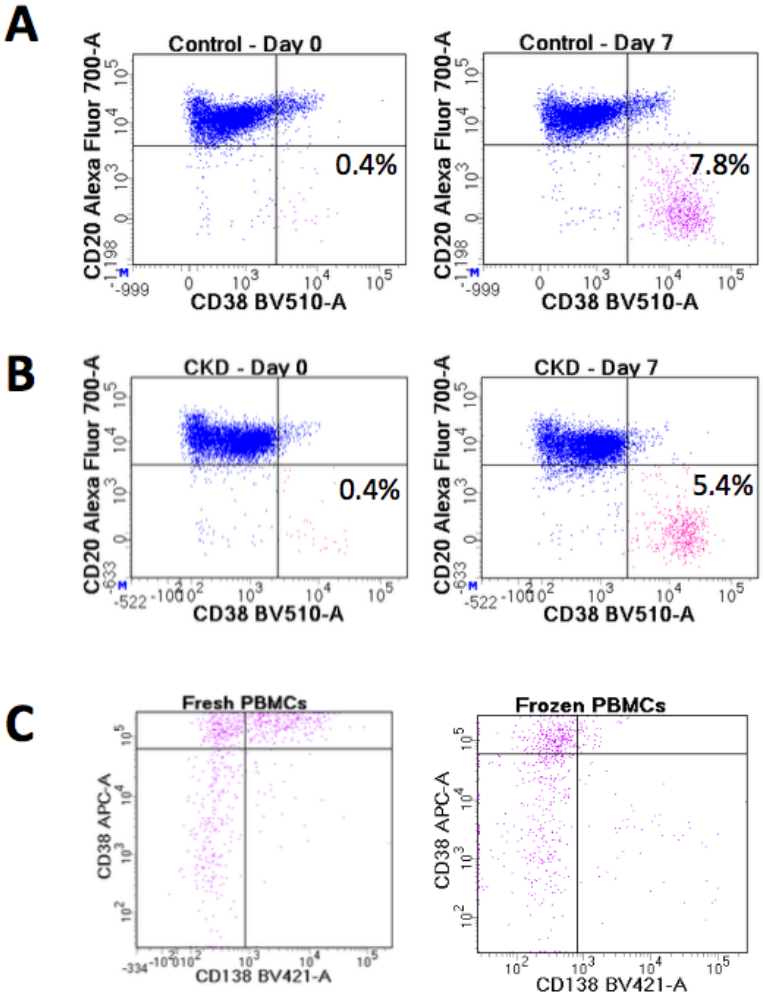
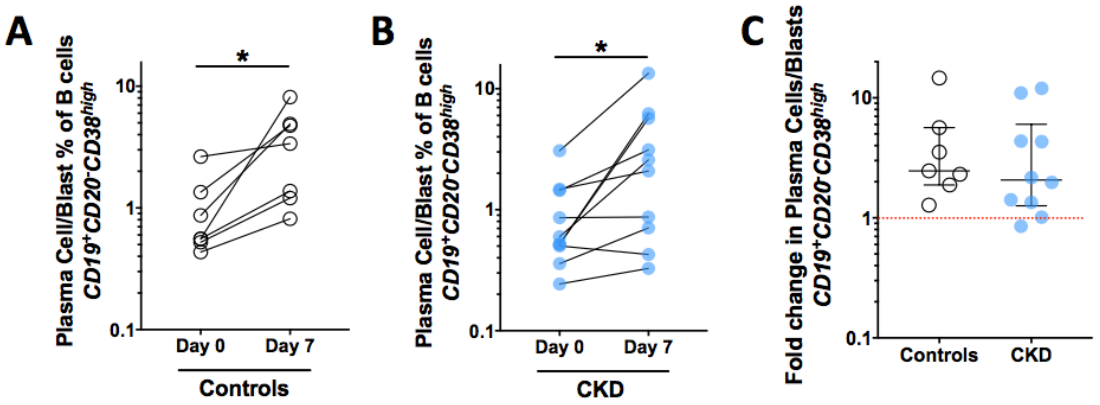


Figure 6-14 Change in circulating plasma cell/blast proportion of B cells from pre-vaccination (day 0) to day 7 post-vaccination.

Change in circulating plasma cell/blast B cell populations in individual controls (A) and patients with CKD (B) from baseline to day 7 after vaccination. Panel C compares the magnitude of change in these populations in patients with CKD and controls. Error bars show median and IQR.

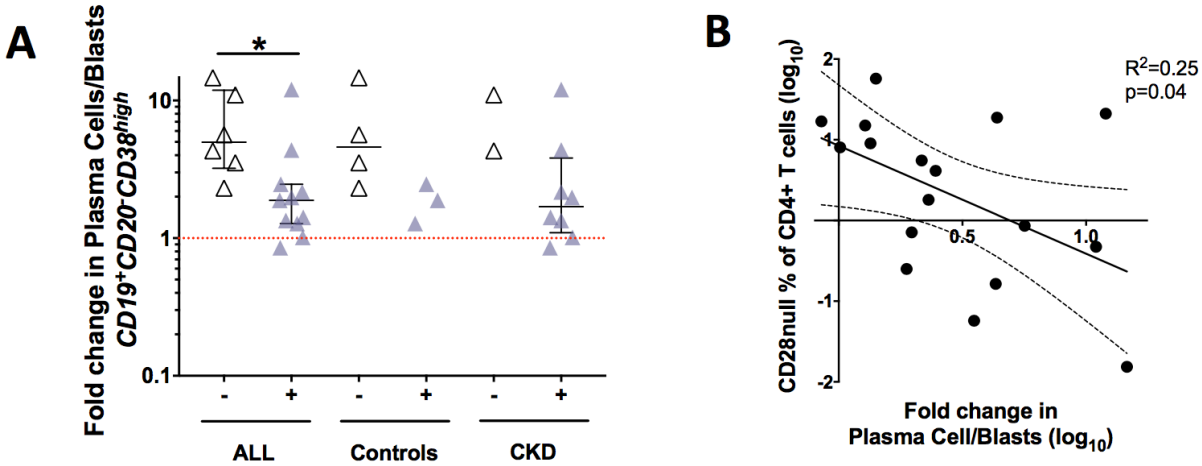
*denotes $p<0.05$



No significant relationships were seen between the fold change in circulating PB/PC proportions of B cells (PB/PC FC) at day 7 and age, CCI, medication burden, eGFR, ACR, HbA1c or hsCRP. Interestingly, CMV seropositivity was associated with lower PB/C FC (Figure 6-15 A) and CMV-specific IgG titre significantly correlated with PB/PC FC (Spearman's R= -0.69, 2-tailed p=0.002). Interestingly, PB/PC FC also significantly inversely correlated with the proportion of CD4+ T cells lacking CD28 (Figure 6-15 B) – a population known to be expanded in latent CMV infection – but not with any other B cell or T cell phenotypes assessed in this study.

Figure 6-15 Impact of latent CMV infection on change in circulating plasma blast/cell populations at day 7 post-vaccination.

A – comparison of magnitude of change in these cell populations between CMV negative and seropositive individuals (error bars show median +/- IQR, dashed red line denotes fold change of 1). B – relationship between the magnitude of change in circulating plasma cell/blasts and $CD28^{null}$ CD4+ T cell proportion (linear regression statistics and best fit line with 95% CI shown) – a phenotype significantly associated with latent CMV infection. *denotes $p<0.05$.



CMV-specific IgG titre (but not the binary variable of CMV serostatus), approached significance as a predictor of PB/PC FC in a linear regression model that also included age, gender and CKD status/CCI (p=0.09). The model fit was not improved with the addition, in turn, of $CD28^{null}$ CD4+ T cell % or measures of multimorbidity. These relationships suggest that latent CMV infection has a significant impact on the generation of plasma cells/blasts at day 7 post-vaccination, independent of renal impairment or multimorbidity.

6.6 B lymphocyte phenotypes associated with vaccine response

Antigen-specific antibody production is the main function of B cells. Hence, I felt it was important to evaluate the relationship between circulating B cell phenotype and measures of vaccine response as described in Chapter 4 (Vaccine response). For brevity and clarity of analysis, I will use the mean ARR for both TIV (TD vaccine) and PPV23 (TI vaccine), which are largely representative of trends seen with individual antigens, as the read-out of vaccine response. The relationships between vaccine response and cross-sectional B cell phenotyping as described earlier in this chapter are summarised in Table 6-1.

Table 6-1 Summary of relationships between B cell populations and vaccine response.
Relationships shown between B cell populations (first column) and TIV mean ARR (orange) and PPV23 mean ARR (blue). Spearman correlation statistics shown. P value <0.05 considered significant – highlighted in bold.

	TIV mean ARR		PPV23 mean ARR	
	Spearman r	p value	Spearman r	p value
B cell %	0.08	0.56	0.26	**0.05**
Naïve %	-0.39	**0.003**	0.002	0.99
IgM memory %	-0.15	0.26	0.05	0.75
Switched memory %	0.34	**0.01**	0.04	0.80
Late memory %	0.31	**0.02**	-0.05	0.72
B_{REG} %	-0.21	0.13	-0.09	0.53
$CD40^+$ %	-0.31	**0.04**	-0.01	0.93
$CD80/86^+$ %	0.09	0.53	-0.03	0.87
$CD40^+CD80/86^+$ %	0.01	0.98	-0.05	0.72

As seen with T cells in Chapter 5 (T cells), there appeared to be a split between the B cell phenotypes associated with robust TIV and PPV23 responses. Higher B cell proportions of total lymphocytes were significantly associated with higher PPV23 mean ARR, but not

mean ARR for TIV (Figure 6-16 A, B). However, higher TIV mean ARR was significantly associated with lower naïve and higher switched and late memory proportions of B cells, whereas no relationships were seen between these variables and PPV23 mean ARR (Figure 6-16 C-H). This could suggest that the magnitude of the humoral response to TIV observed in this study is determined by memory B cell responses, either directed against serotype-specific antigens previously encountered through environmental or vaccination exposure, or directed against conserved influenza antigens, which afford a degree of cross-reactivity between different influenza strains e.g. nucleoprotein and matrix protein 1 (described in Chapter 1, Introduction). This is in keeping with subtle association between mean TIV ARR and expansions of CD8 T cell memory populations described in Chapter 5 (T cells). TIV mean ARR (but not that of PPV23) also negatively correlated with the proportion of B cells expressing CD40 (Figure 6-16 I, J), which may reflect the high expression of this marker on naïve B cells seen in this study. Interestingly, no significant relationships were seen between mean ARR to either vaccine and the proportion of B cells expressing CD80/86 or both CD40 and CD80/86.

In contrast to the associations seen between TIV and PPV23 ARR and cross-sectional lymphocyte phenotypes, the relationships between vaccine response and dynamic changes in circulating plasma blasts/cells (PB/PC FC) following vaccination were similar for TIV and PPV23. PB/PC FC from baseline to day 7 post-vaccination (equivalent between controls and patients with CKD) was significantly associated with both TIV and PPV23 mean ARR (Figure 6-17 A, B).

Figure 6-16 Relationships between vaccine responses (mean ARR) and B cell cross-sectional phenotype.

Relationships shown between TIV mean ARR (orange symbols) and B cell % (A), naïve % of B cells (C), switched memory % of B cells (E), late memory % of B cells (G) and $CD40^+$ % of B cells (I); PPV23 ARR (blue symbols) and B cell % (B), naïve % of B cells (D), switched memory % of B cells (F), late memory % of B cells (H) and $CD40^+$ % of B cells (J). Non-parametric correlation statistics shown. Significance set at 0.05 level – denoted by *.

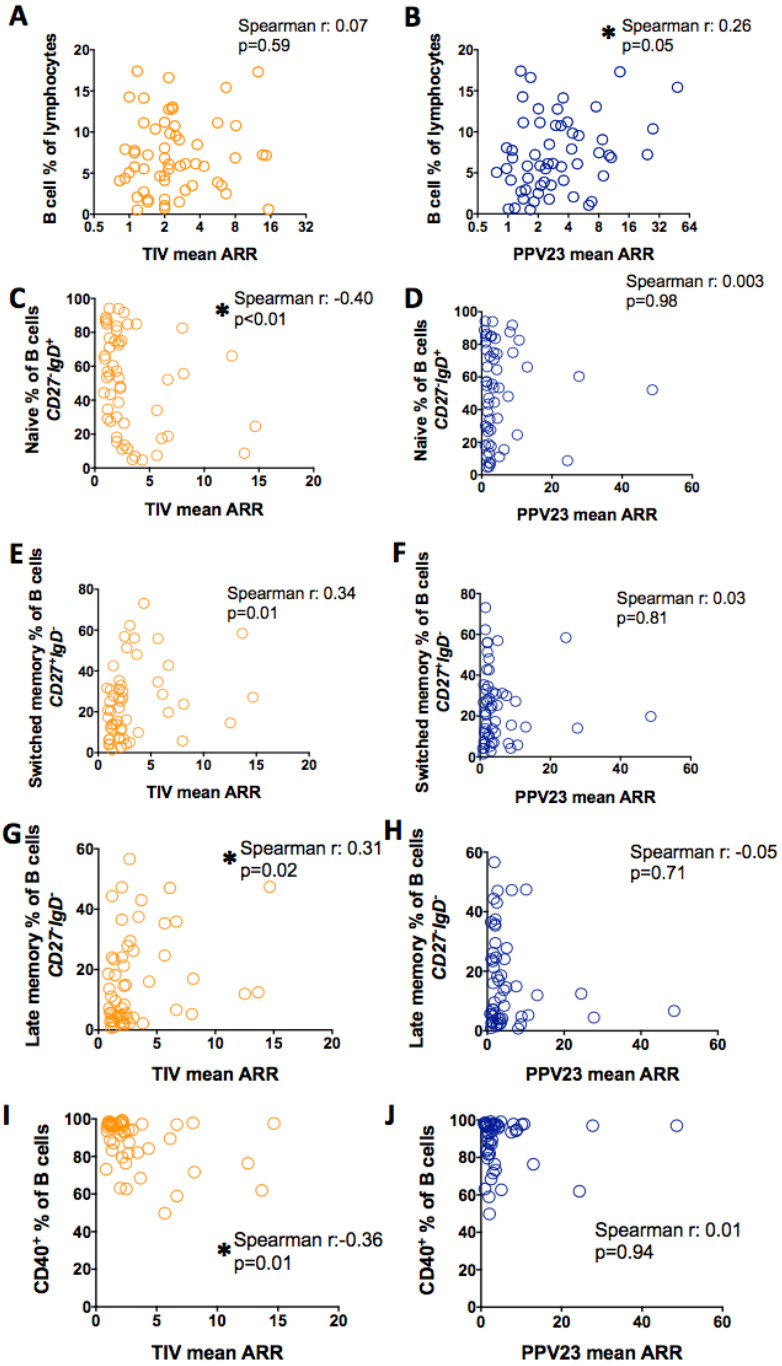
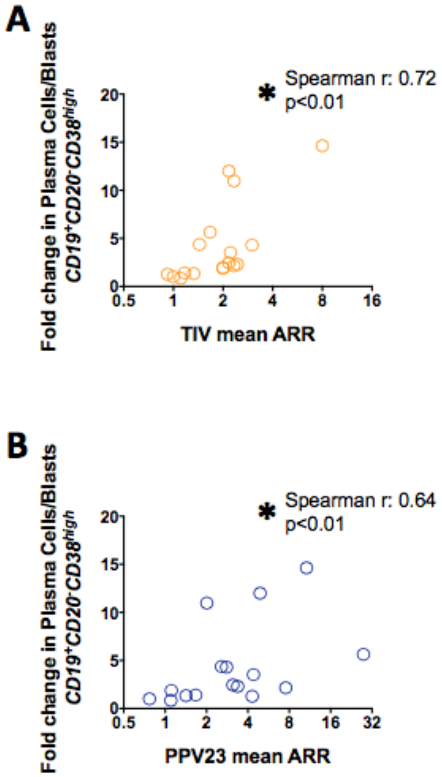


Figure 6-17 Relationships between vaccine responses (mean ARR) and dynamic changes in circulating plasma blasts/cells (fold change from baseline to day 7 post-vaccination) in subset of SONIC participants.

Relationship between fold change in circulating plasma cells/blasts and A – TIV mean ARR (orange symbols), B – PPV23 mean ARR (blue symbols); non-parametric correlation statistics shown. *denotes p<0.05.

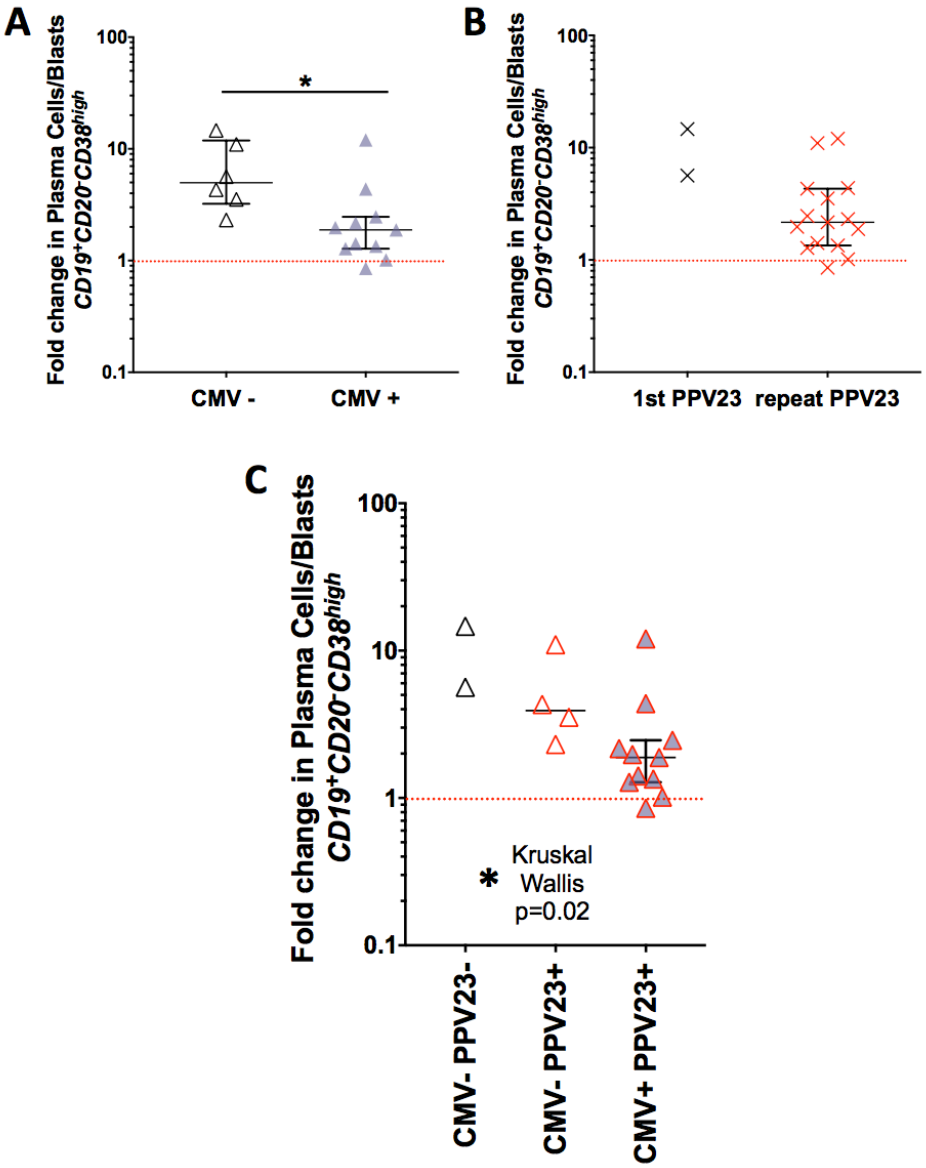


Interestingly, both latent CMV infection and previous PPV23 vaccination status were associated with a smaller PB/PC FC at day 7 post-vaccination (Figure 6-18 A, B) and, unlike what was seen for PPV23 mean ARR, their effect appeared to be additive (Figure 6-18 C). These findings are in keeping with the associations between "senescence"- or CMV-associated T cell phenotypes and TIV mean ARR described in Chapter 5 (T cells). These findings also support the hyporesponsiveness observed in the earlier analysis of

humoral response to PPV23 (Chapter 4, Vaccine response) and highlight the importance of previous PPV23 vaccination in determining subsequent PPV23 response.

Figure 6-18 Effect of latent CMV (A) and previous PPV23 vaccination (B) on dynamic change in circulating plasma cells/blasts at day 7 post-vaccination.

The additive effect of both CMV and previous PPV23 is shown in panel C. Error bars denote median and IQR. +/- denotes CMV seropositive/negative. *denotes p<0.05 (deemed significant).



6.7 Discussion

In this study I have performed a cross-sectional analysis of B cell phenotype in patients with CKD compared to age-matched controls. In line with previous literature (233, 235), I found a reduced circulating number and proportion of B cells in patients with CKD, often reported as a feature of accelerated "ageing" of the immune system. However, due to the marked difference in multimorbidity between controls and patients with CKD, the independent effect of renal impairment here could not be reliably determined. These results are still clinically relevant, as older individuals with non-immune renal disease invariably have multiple comorbidities that are likely to have contributed to the development of renal impairment.

As with previous results, multiple comparisons have been made between data from patients with CKD and controls in this chapter. Although some of the significant findings may represent type I errors (false positives), most are probably due to genuine differences between the disease groups and associations between variables.

Reduced circulating B cell proportion of total lymphocytes was significantly associated with other B cell features previously reported in association with immune ageing (83, 85) when all study participants were evaluated together: contraction of naïve ($CD27^{-}IgD^{+}$) and regulatory B cell proportions ($CD24^{high}CD38^{high}$) and expansion of late memory B cell populations ($CD27^{-}IgD^{-}$). Although patients with CKD were found to have significant reductions in B_{reg} proportions, no significant differences in naïve/memory proportions of B cells were seen between patients with CKD and controls, contrary to previous findings in ESRD (142, 233, 239). This may reflect the inherent immune activating effect of dialysis therapy, rather than renal impairment itself or that the study sample was too small

for a difference to be seen. It could also be that CKD and its associated comorbid state do not affect all aspects of the B cell population equally. Interestingly, expansions of late memory ($CD27^{-}IgD^{-}$) B cells have been associated with chronic immune activation as seen in chronic viral infections such as human immunodeficiency virus (HIV) and hepatitis C (330). The lack of differences in the size of this B cell population between older adults with and without CKD in this study could, therefore, be related to an equivalent immune "imprint" of latent CMV, but this requires further investigation.

Although the $CD24^{high}CD38^{high}$ surface phenotype of B cells has been associated with secretion of IL-10, a canonical regulatory B cell cytokine (85), I cannot confirm this function from the data available in this study. As such, the contraction of the $CD24^{high}CD38^{high}$ population of B cells observed in patients with CKD in this study may represent a reduction in the circulating population of immature B cells, also previously described to have this surface marker pattern (331). This requires further investigation with interrogation of cell function in a larger cohort of patients with CKD.

Patients with CKD in this study had greater proportions of B cells expressing the CD28 ligands CD80/86 than controls. The expression of CD80 or CD86 on B cells has not previously been evaluated in CKD arising from non-immune mechanisms, but one study in dialysis patients has shown downregulation of CD86 expression on circulating macrophages, suggesting the presence of a defect in APC function (332). Indeed, the majority of literature on B cell CD80/86 expression in CKD patients focuses on upregulation of these surface ligands as markers of renal damage and disease activity in systemic lupus erythematosus, such as in references (333, 334), and the use of the monoclonal CTLA4-like antibody, belatacept, which reduces T cell CD28 interaction with

its APC ligands CD80/86, in addition to conventional immunosuppression to improve patient and organ outcomes in renal transplantation (335, 336).

The findings of greater proportions of B cells expressing CD80/86 in patients with CKD than controls are in keeping with what has been observed in some chronic inflammatory states e.g. murine models of inflammatory arthritis (337), but, interestingly, the observations in this study do not correlate with hsCRP. Downregulation of CD4 T cell CD28 was associated with expansion of CD80/86+ proportions of B cells, suggesting that this may be a compensatory mechanism. A novel finding in this study is the effect of both renal impairment and the magnitude of the humoral immune response to latent CMV infection in this upregulation of CD80/86 on B cells, which appeared to be independent of multimorbidity. This is an interesting finding that warrants further investigation.

Despite the phenotypic differences seen in the B cell compartment in patients with CKD, the generation of circulating ASCs (PB/PCs) following vaccination appeared equivalent between patients with CKD and controls in a small sample of the study population. This suggests equivalent differentiation/mobilisation of effector B cells following antigen exposure in patients with CKD compared to controls. The sample size for this particular analysis was small and may not have picked up a difference if it exists, but this is unlikely, given that differences in vaccine antibody responses are marginal between all patients with CKD and controls in this study.

Higher PB/PC FC was significantly associated with the a more robust vaccine response as measured by mean ARR for both PPV23 and TIV. Interestingly, PB/PC FC was significantly altered by the presence of latent CMV infection and previous PPV23 vaccination, with a stepwise reduction in PB/PC FC observed with addition of these two

factors. The presence of hyporesponsiveness to PPV23 has already been observed in the serotype-specific IgG response in this study, as described in Chapter 4 (Vaccine response). The role of latent CMV infection in determining vaccine responses is gaining attention (96, 309) and may represent a therapeutic target in improving immune responses. Indeed, a recent study from our group has shown an effect of therapeutic CMV suppression on responses to a TD vaccine (97).

CHAPTER 7

NEUTROPHIL FUNCTION IN OLDER ADULTS WITH CKD

7.1 Introduction

As described in Chapter 1 (Introduction), few studies have examined neutrophil function in non-dialysis CKD populations and those that have, have shown conflicting results (184-187, 244). In dialysis populations, neutrophils are often described as "primed" or "over-active" and having an exaggerated oxidative burst response to multiple stimuli, but with generally impaired phagocytosis (183, 184). Multiple studies have alluded to the contribution of materials in dialyser membranes to this observation (188, 338), hence it is not clear what the effect of renal impairment *per se* is in these patients.

As neutrophils represent the first line of defence against pathogens, I felt it was vital to examine this cell population in this study of older patients with CKD (who have increased pathogen susceptibility) and also to evaluate the relationship between innate and adaptive immune system defects.

In this Chapter, I will describe results from functional neutrophil assays performed on samples from patients with CKD and age-matched controls. Assessment of neutrophil function evolved during the course of this study. Initially, only assessment of phagocytosis and oxidative burst were performed on whole blood samples (PhagoTest™ and PhagoBurst™ assays), but as differences emerged between patients with CKD and controls, further functional assays were incorporated into the analysis, including generation of NETs and assessment of migration. This approach allowed us to sequentially interrogate different, but related, aspects of neutrophil function in patients with CKD, like peeling back the layers of an onion. The sample size for each functional assessment is presented together with results from that assay in this Chapter and was determined by

practical constraints including availability of donors, volume of blood collected and assay failure.

7.2 Neutrophil phagocytosis is preserved in older adults with CKD

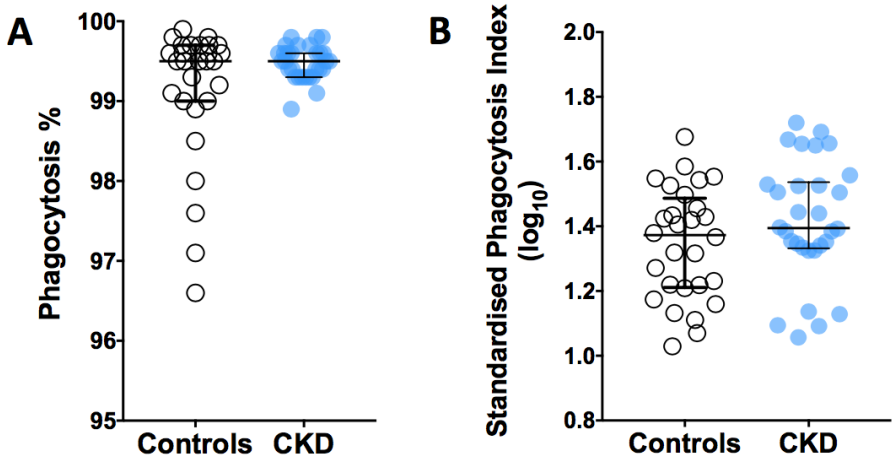
Both neutrophil phagocytosis (PhagoTest™) and oxidative burst (PhagoBurst™) assays (described in Chapter 2, section 2.2.16) were performed in tandem on samples from 65 of 66 SONIC patients. The majority of samples were analysed at the baseline timepoint of the study – 33 patients with CKD and 27 controls, with the remainder (samples from 3 patients with CKD and 2 controls) analysed at the day 7 timepoint due to assay failure at baseline. Exclusion of day 7 samples did not alter the relationships in neutrophil function observed between patients with CKD and healthy controls, hence they are included in the final analysis. Both PhagoTest™ and PhagoBurst™ assays are optimised for whole blood samples with neutrophils in the normal range. Four SONIC subjects (all patients with CKD) had abnormal neutrophil counts on the day these assays were performed (1 low, 3 high) and are, therefore, excluded from the final analysis. One control individual developed pulmonary fibrosis and two patients with CKD were diagnosed with metastatic carcinoma during the course of the study (thus fulfilling exclusion criteria) and their samples were subsequently excluded from analysis of immune function. The final sample size for analysis of PhagoTest™ and PhagoBurst™ assays is 30 patients with CKD and 28 controls.

The proportion of neutrophils ingesting opsonised killed FITC-labelled *E.coli* in the PhagoTest™ assay was equivalent in patients with CKD to that of controls (Figure 7-1 A). The flow cytometer underwent repair after data acquisition for the 2015 cohort, therefore, the MFI in the FITC channel from these patients was not directly comparable to subsequently acquired data. This parameter was, therefore, standardised by dividing the result from stimulated samples by that of the negative control and allowed the phagocytosis index (PI) - the product of the phagocytosing neutrophil % and the FITC MFI of the neutrophil gate – to be calculated for use across the whole SONIC study. As such, no significant difference in PI was observed between patients with CKD and controls, suggesting that the number of bacteria engulfed per cell is equivalent between the two groups. No significant relationships were observed between % phagocytosing neutrophils or PI and eGFR, ACR, hsCRP, CCI or medication burden.

Overall, these results suggest that renal impairment in older adults is not associated with a demonstrable defect in neutrophil phagocytosis of heat killed opsonised bacteria compared to older adults without renal impairment.

Figure 7-1 Results of neutrophil phagocytosis assay (PhagoTest™) – comparison between controls and patients with CKD.

A – proportion of neutrophils undergoing phagocytosis following stimulation with heat killed opsonised FITC-labelled *E Coli*; B –Standardised phagocytosis index (log_{10}) – product of % neutrophils undergoing phagocytosis and standardised FITC MFI of the neutrophil gate. Error bars show median and IQR.



7.3 Reduced neutrophil oxidative burst is associated with declining health status and renal impairment in older adults

Neutrophil oxidative burst (as measured by intracellular oxidation of DHR-123 in the PhagoBurst™ assay) was assessed in response to fMLP, opsonised heat killed *E. coli* and PMA. The bacterial metabolite, fMLP, activates neutrophils through interaction with cell surface G-protein coupled receptors to stimulate superoxide production through activation of NADPH oxidase (339). Opsonised *E. coli* stimulate neutrophils through a number of PRRs, including TLRs and receptors for the Fc portion of immunoglobulin (340). PMA stimulates NADPH oxidase activation through direct stimulation of protein kinase C

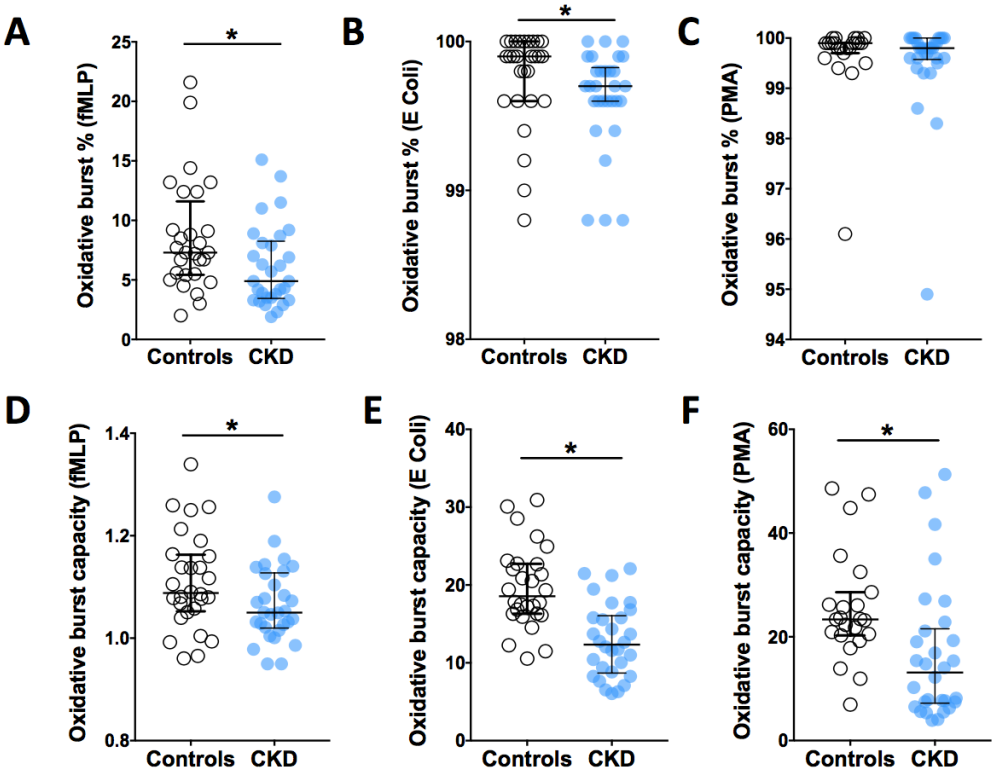
(PKC), without interaction with surface receptors (341). The use of these 3 stimuli in the PhagoBurst™ assay allowed me to test responses to both biological (receptor-mediated) and mitogenic stimuli in neutrophils from the same individual.

The proportion of neutrophils generating reactive oxygen species (hereafter described as "oxidative burst %" for brevity) in response to fMLP and opsonised killed *E. coli* (but not the mitogen PMA) was significantly reduced in patients with CKD compared to controls (Figure 7-2 A-C), suggesting an impairment in receptor-mediated neutrophil activation. Although statistically significant, the absolute reduction in median oxidative burst % in response to *E. coli* was very small in patients with CKD (0.2%) and may not be biologically significant.

However, the neutrophil oxidative burst capacity (defined as FITC MFI of neutrophil gate) was significantly reduced in response to all 3 stimuli in patients with CKD (Figure 7-2 D-F), suggesting that neutrophils from older patients with CKD are not able to generate as much ROS per cell as those from healthy older adults, regardless of the type of activating "signal" they receive. This could limit neutrophil ability to kill ingested bacteria, thus contributing to the increased susceptibility to infection seen in the CKD patient population. FITC MFI has been standardised for use across the entire SONIC cohort by dividing the result from stimulated samples by that of the negative control sample, to account for changes in cytometer settings after data acquisition for the 2015 cohort.

Figure 7-2 Oxidative burst function of neutrophils as assessed by PhagoBurst™ assay.

Proportion of neutrophils from patients with CKD and controls that produced reactive oxygen species (oxidative burst %) is shown in response to A – fMLP, B – *E Coli* and C – PMA. Oxidative burst capacity per cell (standardised FITC MFI) is shown in response to D – fMLP, E – *E coli* and F – PMA. Error bars show median and IQR. *denotes p<0.05.



Oxidative burst % of neutrophils in response to fMLP and *E. Coli* significantly correlated with one another (Spearman r=0.44, 2-tailed p=0.001) and also with eGFR (both MDRD and CKD-EPI eGFR: Spearman r=0.36, 2-tailed p=0.005 and r=0.26, p=0.04, respectively) and CCI (Spearman r= -0.33, p=0.01 for both fMLP and *E. Coli*). Only oxidative burst % in response to fMLP significantly correlated with medication burden (Spearman r= -0.30, p=0.02). No significant correlations were seen between oxidative burst % of neutrophils in response to fMLP or *E. coli* and ACR, hsCRP or HbA1c.

Interestingly, oxidative burst % in response to PMA correlated significantly only with hsCRP (Spearman r= -0.28, p=0.04) and not with measures of renal disease (eGFR, ACR), glycaemic control (HbA1c) or multimorbidity (CCI, medication burden). PMA % response was also not related to oxidative burst % in response to fMLP or *E. coli*, which is not unexpected given their different mechanisms of neutrophil activation.

CCI was a significant independent predictor of oxidative burst % in response to fMLP and *E. Coli* ($p \leq 0.02$) in a linear regression model that also included age and gender. CCI and CKD status/eGFR were significantly collinear and their independent contribution to a multivariate predictive model could, therefore, not be reliably determined. However, the fit of the above linear regression model was not improved by the addition of CKD status or eGFR, suggesting that declining health status was a more prominent predictive factor of neutrophil oxidative burst % in response to fMLP and *E. Coli* than renal impairment alone. No significant predictors were identified when this model was applied to oxidative burst % in response to PMA.

In contrast to oxidative burst %, neutrophil oxidative burst capacity (standardised FITC MFI) in response to all 3 stimuli was significantly associated with eGFR, CCI and medication burden (Table 7-1). Oxidative burst capacity in response only to *E. Coli* was also significantly associated with ACR and hsCRP, and HbA1c was significantly associated with oxidative burst capacity in response to all stimuli except fMLP.

Table 7-1 Relationships between neutrophil oxidative burst capacity in response to the stimuli listed and measures of multimorbidity, renal disease severity, glycaemic control and inflammation.

Correlations between neutrophil oxidative burst capacity to different stimuli (top row) and demographics/laboratory parameters of study participants. R denotes Pearson correlation coefficient, r denotes Spearman correlation coefficient. Statistically significant results highlighted in bold ($p<0.05$).

	fMLP	E Coli	PMA
CCI	***r: -0.28 p=0.03***	***r: -0.58 p<0.0001***	***r: -0.45 p=0.001***
Medication burden	***r: -0.32 p=0.02***	***r: -0.56 p<0.0001***	***r: -0.41 p=0.002***
eGFR MDRD	***R: 0.36 p=0.006***	***R: 0.59 p<0.0001***	***R: 0.38 p=0.006***
eGFR CKD-EPI	***R: 0.34 p=0.008***	***R: 0.59 p<0.0001***	***R: 0.37 p=0.006***
ACR	R: -0.21 p=0.12	***R: -0.45 p<0.001***	R: -0.27 p=0.06
HbA1c	R: -0.16 p=0.22	***R: -0.37 p=0.005***	***R: -0.36 p=0.009***
hsCRP	R: -0.12 p=0.36	***R: -0.26 p=0.05***	R: -0.25 p=0.07

ACR and HbA1c did not have a linear relationship with neutrophil oxidative burst capacity, so were excluded from multivariate analysis. Significant collinearity precluded inclusion of CCI, medication burden and CKD status in the same multivariate analysis, but each variable, in turn, was a significant predictor of neutrophil burst capacity in response to each of the 3 stimuli in a linear regression model that also included age and gender. The best fitting model for responses to fMLP contained medication burden (adjusted R^2 0.04), rather than CCI or CKD status, and the model fit was not improved with subsequent addition of CKD status. The best fitting model for responses to both *E. Coli* and PMA

contained CKD status (adjusted R^2 0.31 and 0.11, respectively) and only the *E. coli* model was improved by the subsequent addition of CCI or medication burden.

This multivariate analysis suggests that, although reduced oxidative burst % and oxidative burst capacity per cell was identified in patients with CKD compared to controls in this study, it is not possible to confidently surmise that this is due to renal impairment alone, outside the context of associated multimorbidity.

7.4 Generation of NETs in response to PMA is reduced in patients with severe CKD compared to healthy controls

The generation of NETs is largely dependent on the production of ROS as a result of NADPH oxidase activation in stimulated neutrophils (342). In view of the oxidative burst defects seen in neutrophils from patients with CKD earlier in the study, I then went on to assess their ability to generate NETs. This fluorimetric assay (described in Chapter 2: Materials and Methods) used the mitogen PMA as the stimulating factor. Although not a true "biological" mimic, responses to PMA (which directly activates NADPH through actions on PKC) give an indication of the capacity of neutrophils to generate NETs after stimulation through other mechanisms, as many intracellular signalling pathways downstream of surface receptors converge on PKC activation (339).

This assay was performed on samples collected at the month 6 study timepoint from the 2016 cohort and the baseline timepoint from the 2017 cohort. These timepoints were considered equivalent in terms of cross-sectional assessment of a quiescent immune state.

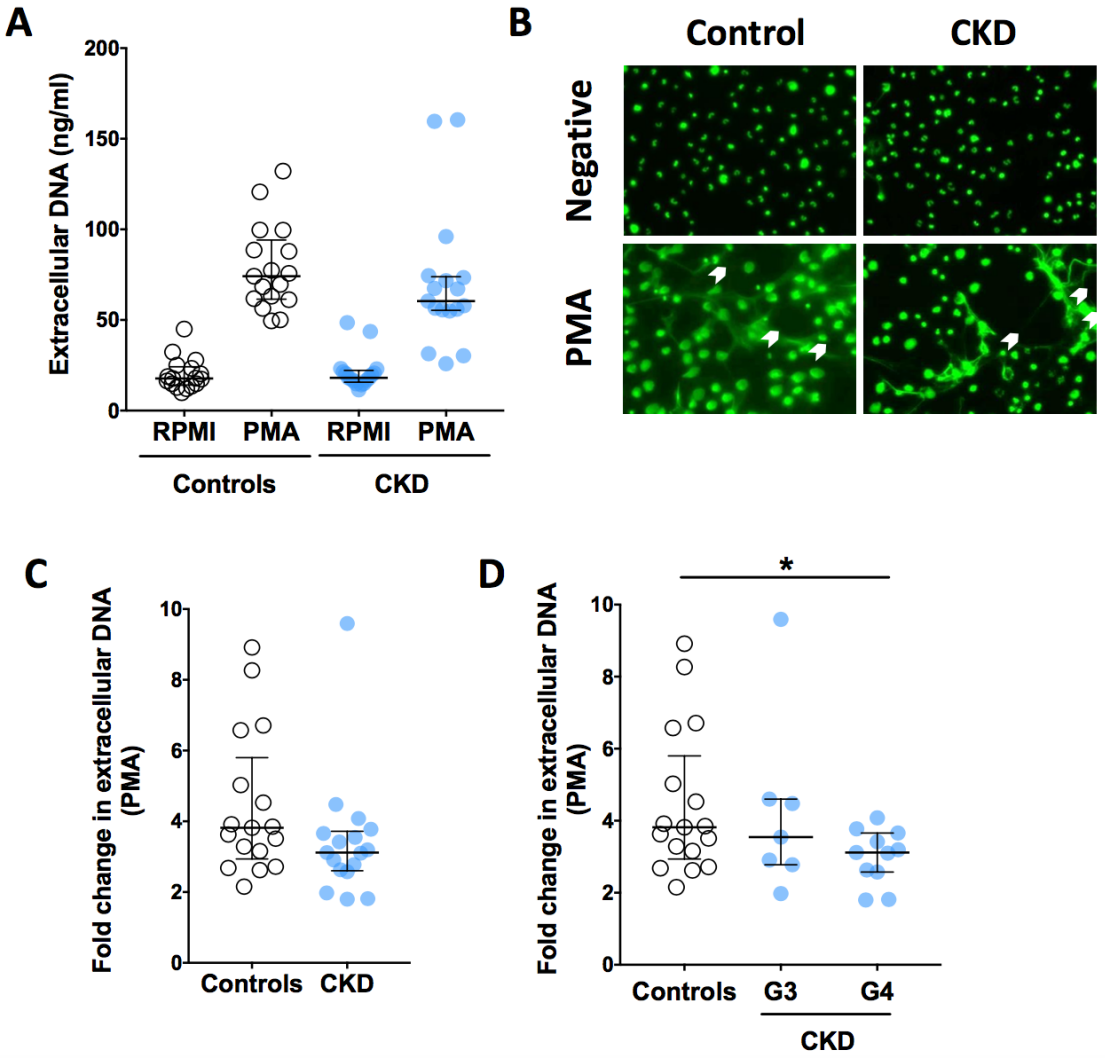
The final sample size for this analysis was 18 patients with CKD and 17 age and gender-matched controls.

There was no significant difference in extracellular DNA concentration (representing NETs) either at background levels (RPMI-PS control) or following PMA stimulation in patients with CKD when compared to controls (Figure 7-3 A). In vitro generation of NETs was visualised using fluorescent microscopy in a sample of patients with CKD and controls (representative images shown in Figure 7-3 B). The fold change in extracellular DNA following PMA stimulation (representative of NETs generation) was overall lower in patients with CKD (Mann Whitney 2-tailed p=0.07, Figure 7-3 C), but with one outlier in the CKD group. When the CKD group was split by renal function into stages G3 and G4 (based on average of eGFR over the first 3 study visits), G4 patients (severe CKD) generated significantly fewer NETs compared to healthy controls (Figure 7-3 D).

The magnitude of NETs generation in response to PMA was significantly associated with eGFR (Pearson R: -0.34, 2-tailed p=0.05 for both MDRD and CKD-EPI eGFR), but not ACR, HbA1c, hsCRP, CCI or medication burden. Interestingly, there were also no relationships between NETs production and any of the oxidative burst indices discussed earlier in this Chapter. This may be due to the small sample size or because of the contribution of other biological processes such as autophagy, now increasingly recognised to be important in NETs generation (343). No significant predictors of the magnitude of NETs generation were identified in a linear regression model that included age, gender and CKD status/eGFR.

Figure 7-3 Neutrophil generation of NETs in patients with CKD and controls.

A – extracellular DNA (NETs) at background and after PMA stimulation; B – representative fluorescent microscopy images of neutrophils from patients with CKD (x20 magnification) - NETs marked with white arrows; C – fold change in extracellular DNA (NETs): split by disease group; D – fold change in extracellular DNA (NETs) compared with controls by CKD severity. Error bars show median and IQR. *denotes Mann Whitney 2-tailed $p<0.05$.



7.5 Reduced neutrophil migration towards fMLP, but not IL-8 in patients with CKD

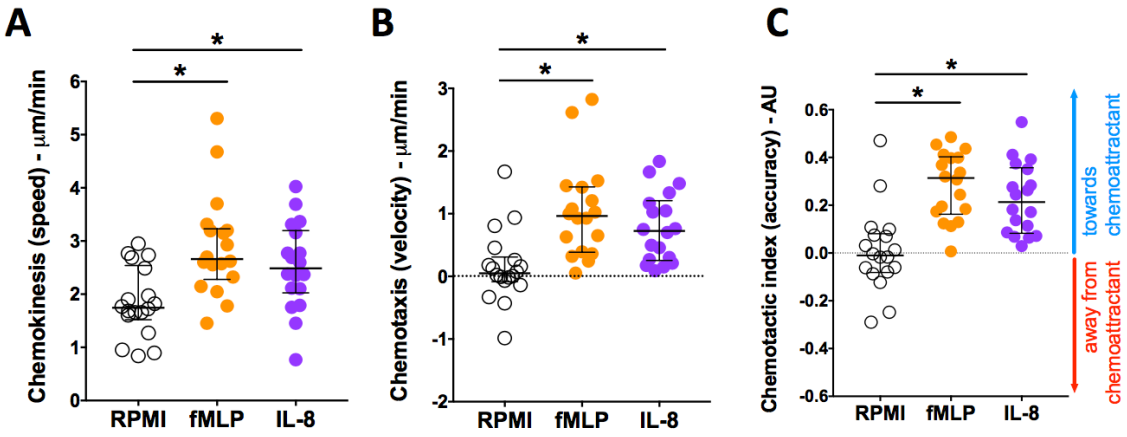
The bacterial metabolite fMLP can stimulate different functions in neutrophils depending on the concentration of fMLP the cell is exposed to. Two different fMLP receptor subtypes exist, which activate different intracellular signal transduction pathways. Low fMLP concentrations activate intracellular signalling responsible for the chemotactic response through interaction with the high affinity formyl peptide receptor (FPR). High fMLP concentrations (as seen at sites of infection) stimulate signal transduction that leads to superoxide production and release of lysozyme through interaction with the low affinity receptor subtype: FPR like 1 (FPRL1) (339). In view of the impaired oxidative burst following fMLP stimulation seen in neutrophils from patients with CKD earlier in the study, I then went on to investigate neutrophil chemotaxis to fMLP. I also used the chemoattractant IL-8 (CXCL8) to evaluate neutrophil migration to a receptor-mediated inflammatory, rather than a microbial stimulus (344).

Analysis of neutrophil migration was performed on samples collected from 10 patients with CKD and 8 age and gender-matched controls from the 2017 cohort, at either day 28 or month 6 post-vaccination (considered equivalent in terms of a quiescent neutrophil state).

Gradients of fMLP and IL-8 significantly altered migration of neutrophils from all individuals tested when compared to the negative control (RPMI) – Figure 7-4, showing induction of stimulation with the reagents used.

Figure 7-4 Neutrophil migration results from all individuals tested towards RPMI (negative control), fMLP and IL-8.

A – chemokinesis (speed); B – chemotaxis (velocity); C – chemotactic index (accuracy). Error bars show median and IQR. *denotes Mann Whitney 2-tailed p<0.05. These results demonstrate that reagents used in the assay significantly altered neutrophil migration.

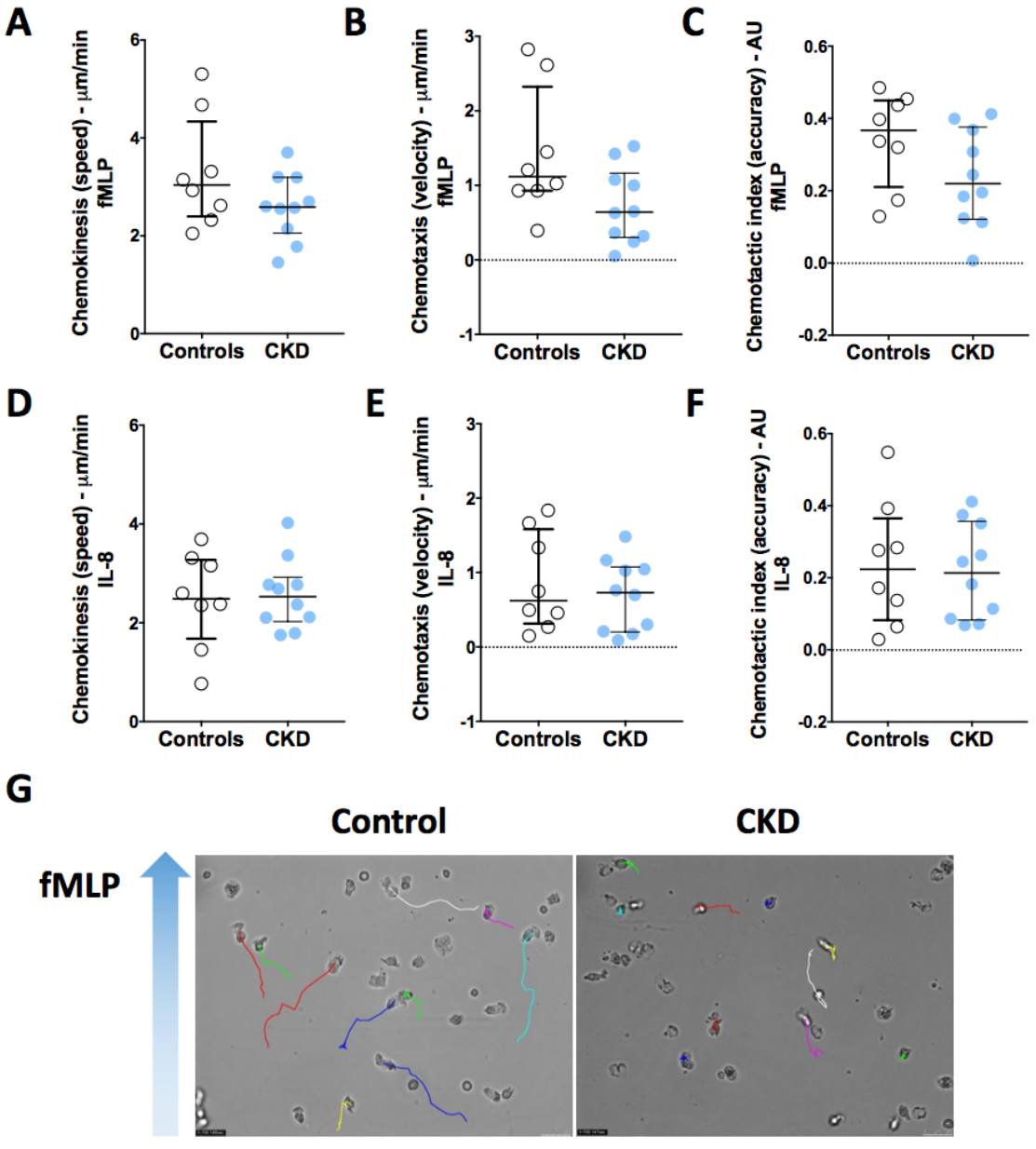


Slightly reduced velocity (chemotaxis) and accuracy (chemotactic index, CI) of neutrophil migration towards fMLP (Mann Whitney 2-tailed p=0.08 and 0.12, respectively; Figure 7-5 B, C and G), but not IL-8, were seen in patients with CKD compared to healthy controls (Figure 7-5 D-F).

Accuracy of neutrophil migration (CI) towards fMLP, but not IL-8, was significantly associated with degree of proteinuria (ACR, Spearman r: -0.55, p=0.02) and medication burden (Spearman r: -0.51, p=0.03). CCI, eGFR, HbA1c and hsCRP were not significantly associated with either fMLP or IL-8 CI.

Figure 7-5 Neutrophil migration – comparison between patients with CKD and controls.

A-C: migration indices in response to fMLP; D-F: migration indices in response to IL-8; E: representative cell tracking plots for patients with CKD and controls in response to fMLP (brightfield microscopy, x40 magnification). Error bars show median and IQR.



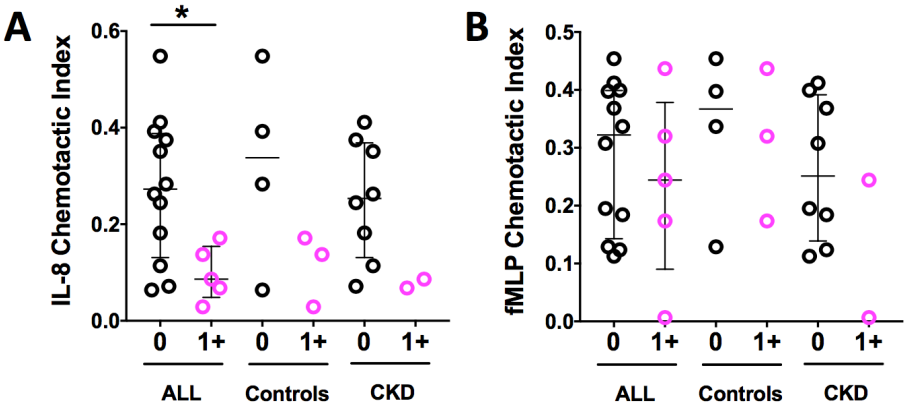
Medication burden was a significant predictor of neutrophil migratory accuracy (CI) to fMLP, independent of age and gender in a linear regression model (p=0.01). The model fit was not improved with the addition of ACR or eGFR (collinear with medication burden), suggesting that polypharmacy was a stronger predictor of neutrophil migration accuracy towards fMLP than measures of renal disease.

Although the sample size for this analysis is small, the findings suggest reduced neutrophil responsiveness to stimulation by bacterial metabolites in older adults with CKD and other comorbidities, compared to healthy older adults.

Interestingly, although no significant differences were seen in neutrophil phagocytosis, oxidative burst response/capacity or NETs generation between individuals reporting infections and those that did not, neutrophil migratory accuracy (chemotactic index) towards IL-8 (but not fMLP) was significantly lower in those who did report infections during the course of the study (Mann Whitney 2-tailed p=0.03, Figure 7-6).

Figure 7-6 Accuracy (chemotactic index) of neutrophil migration towards IL-8 (A) and fMLP (B) – comparison between individuals that reported 1 or more infection.

Pink symbols denote individuals reporting one or more infections (+1) and black symbols denote those that did not (0). Error bars show median +/- IQR. *denotes Mann Whitney 2-tailed p<0.05.



7.6 Discussion

Overall, the results presented in this Chapter represent a comprehensive assessment of multiple aspects of neutrophil function in a well-characterised cohort of patients with CKD and age and gender matched controls.

Phagocytosis of heat killed opsonised *E. coli* was similar in neutrophils from older adults with CKD compared to cells from age-matched controls, contrary to previous literature in ESRD populations (183, 184). This may reflect the inherent effect of dialysis therapy in driving neutrophil dysfunction (which was excluded from this study) and/or differences in experimental techniques. Indeed, a recent study using the PhagoTest^{TM} assay method in non-dialysis CKD patients showed similar results to SONIC (194).

Several functional defects that could result in reduced pathogen clearance were identified in patients with CKD: reduced proportion of cells generating ROS in response to physiological stimuli (fMLP and *E.* coli, although the biological effect of the small relative reduction to the latter may not be significant), reduced oxidative burst capacity in response to both physiological and mitogenic stimuli, reduced capacity to generate NETs in severe CKD (stage G4) in response to PMA and reduced migratory accuracy towards the bacterial metabolite fMLP. All but one of these parameters (migratory accuracy) were significantly associated with reducing eGFR, with variable inverse associations with ACR, a marker of kidney damage. However, due to the difference in multimorbidity between the two study groups, the effect of renal impairment alone cannot be reliably elucidated. Indeed, decreasing health status (measured using either CCI or medication burden) is the strongest predictor of neutrophil dysfunction in this study.

Previous studies of neutrophil oxidative burst function in CKD or dialysis have shown both increased and decreased function. These variations may be explained, in part, by immune activating effects of dialysis therapy, variations in patient selection and different experimental techniques. The findings of this study suggest that previously observed increased neutrophil ROS burst in ESRD patients may, indeed, be a treatment effect. Interestingly, a recent study examined neutrophil oxidative burst in non-dialysis CKD patients using the PhagoBurst™ assay, but the authors report only equivalent oxidative burst % responses to PMA (which is also seen in this study) and not the physiological stimuli included in the assay kit, without analysis of oxidative burst capacity per cell (MFI), concluding that neutrophil oxidative burst function is preserved in CKD.

Literature on neutrophil migration in non-dialysis CKD is limited, but impaired neutrophil migration towards fMLP was recently reported following treatment of cells with FGF23 – a key biomarker associated with CKD (345) and another elegant study elegantly demonstrated FGF23-mediated impairment of selectin-mediated slow rolling and chemokine-induced neutrophil migratory arrest (essential for neutrophil movement out of the vasculature and into inflamed tissue) in non-dialysis patients with CKD (346). The findings of this study are in keeping with this literature, but I did not investigate the contribution of FGF23.

Interestingly, individuals reporting infections during the course of this study had lower neutrophil migratory accuracy to IL-8. This finding is comparable to that seen in patients with COPD who have a greater susceptibility to respiratory infections (347).

Other systemic non-autoimmune chronic inflammatory disease states e.g. COPD have been associated with a number of neutrophil functional defects, including impaired

migratory accuracy and reduced generation of NETs (72), mirroring the findings of this study in patients with CKD (a systemic disease associated with chronic inflammation). This may suggest that chronic low-grade inflammation, regardless of origin, could be driving neutrophil dysfunction. There were, however, no significant relationships between the inflammatory marker hsCRP and neutrophil function parameters in this study. I did not measure systemic levels of other pro-inflammatory cytokines e.g. IL-6 or TNF-α, which may have yielded a different relationship to that seen with hsCRP. However, in contrast to the findings of this study in patients with CKD, studies of oxidative burst in neutrophils from patients with COPD have shown enhanced production, potentiating excessive tissue damage associated with infection and neutrophil phagocytosis is often impaired (72).

As described in Chapter 1 (Introduction), neutrophil function also declines with age. Neutrophils from older adults frequently exhibit reduced phagocytosis, reduced killing ability, impaired NETs generation (74) and impaired chemotactic accuracy (75). As such, neutrophil dysfunction seen in this study of patients with CKD is largely in keeping with defects reported in association with chronological ageing, supporting the accelerated ageing hypothesis of CKD.

Although several functional defects were found that could contribute to reduced pathogen clearance, this was not formally tested in a cell-mediated bacterial killing assay, which could yield further insights into neutrophil dysfunction associated with CKD. The sample size of the whole study is relatively small and the sequential addition of new functional assays reduced this further, thus limiting the power of the analysis. As such, confirmation of the findings observed in this study should be sought using a larger cohort of patients with CKD and age-matched controls. Further insights into the nature of and mechanisms

underlying neutrophil dysfunction in CKD could also be gained from assessment of neutrophil phenotype and quantification of immature granulocytes (shown to be a major contributor to neutrophil dysfunction associated with sepsis and thermal injury (259)), which was not performed in this study.

Several groups have proposed environmental factors such as the retention of various uraemic solutes as a possible mechanism for neutrophil dysfunction observed in patients with CKD (182). However, without a complete understanding of the nature of neutrophil dysfunction, it is not possible to confirm the responsible mechanism. Also, as reviewed elegantly in (72), current literature on neutrophil function in advancing age and chronic illness is prospective and it is unclear which factor came first: the altered neutrophil function, the chronic illness, the ageing or the low-grade inflammation.

As with previous results, multiple comparisons have been made between data from patients with CKD and controls in this chapter. Although some of the significant findings may represent type I errors (false positives), most are probably due to genuine differences between the disease groups and associations between variables.

CHAPTER 8

GENERAL DISCUSSION

8.1 Major study findings and contribution to understanding of CKD-associated immune dysfunction

In this thesis I have described the findings of a prospective observational study of the immune system and responses to external antigen challenge (vaccination) in older adults with and without chronic kidney disease.

Contrary to previous literature documenting poor vaccine responses associated with ESRD (171, 179), only subtle reductions in the magnitude of the antibody increase at day 28 after vaccination with TIV and PPV23 were seen in patients with CKD compared to controls in this study. This may be due to the small sample size of the study, but also that a difference does not exist. Indeed, different studies report variable TIV and PPV23 responses in adults with CKD (176, 178, 179, 277, 278). The strongest evidence for a reduced vaccine response in the CKD population is with the hepatitis B vaccine (HBV) (179). The most common HBV vaccine in use is Engerix B (GSK, Belgium), which is adjuvanted with alum, unlike seasonal TIV, which is not. Therefore, although both vaccines are T-dependent, the mechanics of immune responses elicited to HBV vaccination are likely to be different to that seen with TIV. Also, the use of TIV and PPV23 as surrogates for natural pathogen encounter in this study may be flawed as they represent a suboptimal mimic of natural infection. The responses seen with specific "narrow" antigen exposures in CKD may be largely preserved and significant immune defects may be revealed with activation of the whole immune response as occurs with natural infection. Indeed, despite the small sample size, patients with CKD did report a significantly greater number of respiratory infections than controls, in the face of only subtle differences in humoral vaccine responses. As such, future studies of immune responses in CKD could use

vaccines adjuvanted with agonists of the innate immune system (e.g. TLR9 (348, 349)) or live attenuated vaccines e.g. *Salmonella* Typhi Ty21a (350) as a better mimic of natural infection.

An important finding in the response to PPV23 in this study was hyporesponsiveness to repeat vaccination, with reduced mean ARR in both controls and patients with CKD who had previously received PPV23, even with a median time lag of 10 years. This phenomenon has been reported previously, but its clinical relevance remains under debate, as discussed earlier in this thesis. However, the finding of Pn-specific IgG titre reduction in 8 out of 12 serotypes tested to below pre-vaccination levels at month 6 in 40% of PPV23 revaccinees in this study suggests that repeat PPV23 vaccination may not be beneficial in older adults with or without chronic disease. Previous studies have shown only marginal reductions in morbidity and mortality with PPV23 vaccination in older adults (281) and the findings of this study suggest that this may need to be revisited in the post-PCV era.

8.1.1 Lymphocyte phenotypes

Cross-sectional analysis of lymphocytes in this study revealed a preserved T cell proportion of lymphocytes, but a global B lymphopenia in patients with CKD compared to controls, in keeping with previous findings (215). No significant CKD-associated differences in naïve/memory T or B cell phenotypes were seen, in contrast to previous studies (182). This may be due to the small sample size in this study or that the difference does not exist. Unexpectedly, the size of circulating “senescence”-associated T cell populations (defined by loss of surface CD27/28 and/or gain of CD57/KLRG1) was

similar between patients with CKD and controls, which is not in keeping with the hypothesis of accelerated immune ageing in CKD.

I also demonstrated an expansion of "Th2-like" populations of T cells in patients with CKD compared to controls, contrary to several (but not all (226, 227)) previous studies that report a polarisation towards a Th1 phenotype (182, 224). This could be due to the heterogeneity in experimental methods used e.g. surface chemokine receptor phenotyping used in this study, compared to intracellular transcription factor analysis and/or functional assays performed by other groups. On a physiological level, dialysis therapy rather than renal impairment itself may drive Th1 polarisation (182, 224), explaining this finding in studies of patients with ESRD.

T_{reg} populations were seen to be expanded in patients with CKD compared to controls in this study, independent of CMV. Previous studies have reported both increased and decreased circulating T_{regs} in association with CKD (221, 229, 230). Expansions of T_{regs} may induce excessive immunosuppressive effects, thus impairing immune responses to antigen and resulting in greater susceptibility to infection.

Contraction of the B_{reg} population in patients with CKD in this study is a novel finding and supports the hypothesis of accelerated immune ageing, as these features have previously been described in healthy older adults (85). Although antigen-specific cellular responses were not comprehensively interrogated in this study, I did demonstrate an equivalent expansion of circulating plasma cells/blasts at day 7 post-vaccination in both patients with CKD and controls, which significantly correlated with higher day 28 ARRs to both vaccines. This is a novel finding in CKD and suggests preserved capacity to generate ASCs to TIV/PPV23, although this requires confirmation in a larger cohort.

8.1.2 Contribution of latent CMV infection to lymphocyte phenotype and vaccine response

The dominance of latent CMV infection, rather than health status, on shaping the memory T cell phenotype in both patients with CKD and controls is an important finding. Although expansion of $CD28^{null}$ T cells and the late differentiated T_{EMRA} populations are known to be driven by chronic antigen exposure as seen in latent CMV infection (88), the interaction between CMV and chronological ageing/associated comorbidity is still debated. The lack of difference in relative "senescence"-associated T cell populations (e.g. $CD28^{null}$, $CD57^{+}KLRG1^{+}$, T_{EMRA}) seen in this study between patients with CKD and controls, may be due, in part, to a similar magnitude of the immune effect of CMV in the two groups. Previous studies demonstrating expansions of $CD28^{null}$ T cell populations in patients with CKD, independent of CMV, have frequently assessed the presence of the virus in a binary fashion, without quantitative assessment of CMV-specific IgG (217). In this study, CMV seropositive individuals with CKD and controls had equivalent titres of CMV-specific IgG, which is frequently used as a surrogate of the CMV "imprint" on the adaptive immune system (95). Previous studies may not have "controlled" for the magnitude of CMV-associated effect, which could manifest as a difference between controls and individuals with CKD.

Interestingly, our group has previously shown higher CMV-specific IgG titres in a younger cohort of CMV seropositive adults with CKD compared to age-matched controls, suggesting an effect of CKD on CMV immune responses (180). As it is unlikely that latent CMV infection directly causes CKD, one explanation for this observation is that CKD-

induced immune dysfunction results in impaired host control of CMV infection, resulting in increased frequency of subclinical reactivation and hence, increased antigen exposure, which drives the expansion of CMV-specific memory T cell populations to levels greater than seen in the general population. A possible explanation for the lack of difference seen in CMV-specific IgG titres between the disease groups in this study is that CKD could a different effect on the immune system depending on the age of the host, in keeping with the observation of reduction in the magnitude of relative risk increase of infection between individuals with CKD and the general population with increasing age. Latent CMV infection is also associated with Th1 polarisation of $CD4^+$ T cells and previous studies have not adequately controlled for this (302, 351). The parity of CMV-associated immune "imprint" between the disease groups in this study may also account for the findings of "Th2-like" cell expansions in patients with CKD, contrary to previous data. As such, future studies of lymphocyte phenotype and function in patients with CKD should not only consider the presence of latent CMV infection, but also the magnitude of the CMV-specific humoral response.

Another novel and important finding is the effect of latent CMV infection on vaccine responses. CMV seropositivity was associated with lower day 28 mean ARR for PPV23 in all study participants, with previous PPV23 vaccination adding only a small extra reduction in responses in these individuals. Expansions of CMV-associated T cell populations (e.g. $CD28^{null}$) were also associated with poorer TIV responses as measured using day 28 mean ARR. Latent CMV infection and previous PPV23 vaccination appeared to have an additive effect on reducing the magnitude of plasma blast/cell expansions at day 7 post-vaccination, even when examined in a small sample of study participants. These findings suggest that latent CMV infection could play a greater role in

impairment of immune responses seen in older adults with and without chronic disease than previously thought. Indeed, a recent study from our group in vasculitis patients (some with renal impairment) showed improved responses to PCV13 with reduced CMV reactivation episodes following 6 months of antiviral treatment (97).

In addition to CMV, latent infections with other viruses, e.g. Epstein-Barr virus (EBV), herpes simplex virus (HSV), varicella zoster virus and polyomaviruses, are more prevalent with increasing age. Indeed, an estimated 5-10 individual viruses make up the persistent or chronic virome in adults (352, 353). Individual players in this persistent virome exhibit different cellular tropisms for latency. For example, CMV reservoirs (e.g. haematopoietic stem cells, monocytes/macrophages, vascular endothelium) are located throughout the entire body, with systemic potential for reactivation, whereas HSV is limited to craniofacial and/or anogenital sensory neurones and exhibits localised reactivation in the immunocompetent host (353, 354). Despite these differences, there is increasing evidence that viral reactivation at a local level can alter systemic immune responses (353-355). As such, it is possible that the effects on the adaptive immune system seen in association with latent CMV in this study are also impacted on by other latent viral infections, or a common factor that predisposes an individual to multiple latent viral infections.

8.1.3 Neutrophil function

In this study I have identified multiple functional defects in neutrophils from patients with CKD, including reduced oxidative burst capacity to multiple stimuli, together with reduced generation of NETs and impaired migratory accuracy to fMLP (which failed to reach statistical significance, possibly limited by sample size). As neutrophils form the

first line of defence against microbial invasion, these features may contribute the observed increased susceptibility to infection.

In contrast to previous reports of impaired neutrophil phagocytosis in ESRD (183, 185), I found no difference in the phagocytic capacity of neutrophils between individuals with mild-moderate CKD and controls. Oxidative burst in neutrophils from patients with CKD has previously been reported to be both increased (184, 186) and decreased (187), with significant variations between studies in terms of experimental techniques used and participant selection. Interestingly, a recent study (194) using the same experimental technique (PhagoBurst© assay) reported preserved responses to PMA in terms of proportions of neutrophils producing ROS (similar to findings of this study), but did not interrogate this further through analysis of oxidative burst capacity per cell (MFI), where the most striking differences between controls and patients with CKD were seen in this study.

Impaired neutrophil migration towards fMLP was recently reported following treatment of cells with FGF23 – a key biomarker associated with CKD (345), supporting the findings of this study. Interestingly, individuals reporting infections during the course of the study had significantly lower neutrophil migratory accuracy to IL-8, even in this small study sample. This finding is comparable to that seen in patients with COPD who have a greater susceptibility to respiratory infections (72).

The neutrophil dysfunction seen in this study of patients with CKD is largely in keeping with defects reported in association with chronological ageing (72), supporting the accelerated immune ageing hypothesis of CKD. Several groups have proposed environmental factors such as the retention of various uraemic solutes as a possible

mechanism for neutrophil dysfunction observed in patients with CKD (182). Indeed, the uraemic milieu (particularly the presence of FGF23) has been shown to alter function of healthy neutrophils (345, 346). Another possible mechanism for CKD-associated neutrophil dysfunction is an alteration in cell metabolism. Indeed, mitochondrial dysfunction has previously been demonstrated in CKD (356). Overall, further comprehensive assessment of neutrophil function and phenotype in larger cohorts of patients with CKD is required to fully characterise the nature of CKD-associated neutrophil effects.

8.2 Strengths and weaknesses

This study has several strengths. Patients with CKD were well controlled for age and gender and the study selection criteria excluded several potential confounders, including dialysis therapy and immunosuppressive/modulatory comorbidities. Although the significantly higher prevalence of DM seen in patients with CKD than controls is a potential confounder in the analysis of CKD impact on the immune system in this study, the findings and conclusions remain highly applicable to the older population of CKD patients, who are usually multimorbid. As such, CKD and its associated comorbidities in older age could be considered a phenotype of "unhealthy ageing" and insights gained from this study may be relevant to the study of immune processes in "healthy ageing". Another key strength of this study is the concurrent assessment of multiple aspects of the innate and adaptive immune systems in the same individual and in the context of clinical data such as infection incidence over the course of the study.

The main weakness of this study is the small overall sample size. Although the recruitment target of 100 individuals was achieved, a substantial number of participants withdrew from the study prior to vaccination, much of which was beyond my control. I also found that the older, comorbid individuals in my target CKD recruitment pool were frequently reluctant to commit to extra hospital visits, due to transport difficulties and multiple already scheduled hospital visits. However, the sample size of this study is representative of previous literature (214, 239). The study population was mostly of White ethnicity, and therefore findings may not be applicable to more ethnically diverse CKD populations.

Multiple comparisons have been made between data from patients with CKD and controls in this thesis. Although some of the significant findings may represent type I errors (false positives), most are probably due to genuine differences between the disease groups and associations between variables.

Although the study design eliminated a number of clinical confounders, I did not assess levels of vitamin D, hepcidin and FGF23 (previously shown to be associated with immunomodulation in CKD). I also did not assess the systemic inflammatory milieu beyond characterisation of hsCRP levels, but it is unlikely that pro-inflammatory cytokine characterisation in this study would have added significantly to what is already known in CKD, particularly as it would not be possible to identify the cell types responsible for their secretion.

Weaknesses of the evaluation of humoral vaccine response in this study include lack of functional assessment of antibody produced e.g. affinity, avidity and opsonisation capacity, together with the lack of characterisation of antigen-specific IgA, IgM and

subclasses of IgG produced, which could yield further insights into the phenotype of the immune response to TIV/PPV23 in older adults with CKD. However, in relation to antibody functionality in CKD, previous work from our group has shown that cell-independent serum killing of *Salmonella* Enteritidis is preserved in older patients with CKD compared to controls, suggesting that this patient population are able to make functional antibody (180).

A weakness of the evaluation of lymphocytes in this study is the lack of assessment of cellular function, including proliferation and cytokine secretion profiles following stimulation, together with assessment of T_{reg} and B_{reg} suppressive capacity.

8.3 Future perspectives

The work presented in this thesis could be extended in several ways, including the evaluation of lymphocyte function (as described above) in addition to phenotype. The hypothesis of "uraemic milieu-mediated" neutrophil dysfunction (and its potential mechanisms) could also be explored through assessment of alterations in function of neutrophils isolated from healthy young individuals that are treated with serum/plasma from patients with CKD in this study.

In view of the observed significant CMV-associated immune effects in this study, further work in the field of CKD-associated immune dysfunction could evaluate this in more detail, for example in a larger cohort of patients with CKD and controls. Given recent findings of improved TD vaccine responses following valacyclovir treatment in an immunosuppressed patient cohort, future work could also investigate the effect of CMV

suppression on vaccine responses/incidence of infection in patients with non-inflammatory CKD. The quantification of CMV reactivation e.g. urine CMV PCR in subjects recruited to this study could yield further insights into the biology of CMV latency and its effect on the adaptive immune system in health and disease. As discussed earlier, CMV represents only one of a number of possible players in the latent human virome that have potential to shape immune responses. Further work in this study could characterise the full extent of the latent virome in each individual and evaluate their virus-specific immune responses.

As highlighted earlier, TIV and PPV23 may not represent adequate mimics of natural infection, therefore future studies should consider using other vaccines, responses to which are known to more closely mimic natural infection (e.g. live attenuated), or the concomitant use of agents that stimulate innate immune responses (e.g. CpG DNA – a TLR9 agonist).

Given the complex and important interplay between innate and adaptive immune systems,

further work in characterising the immune dysfunction associated with renal impairment should include assessment of APCs. The function of professional APCs such as DCs is key to generating effective and long-lasting memory responses to antigen. DCs have previously been largely examined in dialysis populations with reported depletion of circulating plasmacytoid dendritic cells (which express TLR7 and TLR9) and dysfunction, both of which are transiently exacerbated by haemodialysis treatment (293). As such, the effect of renal impairment alone on DCs remains unclear.

It is also important to note that the study of immune cell populations in CKD has largely been limited to evaluation of peripheral cells, without assessment of structural or organisational changes in secondary lymphoid organs e.g. lymph nodes, or in bone

marrow. Further insights into the nature of CKD-associated immune dysfunction may be gained by examination of immune cell interactions in lymph nodes and immune cell populations in bone marrow, for example long-lived plasma cells. Recent studies have shown local immunomodulatory effects of adipose tissue on B cell populations (357), which may also be of interest in CKD, where adipokines such as leptin are thought to contribute to the chronic inflammatory state.

The characterisation of immune dysfunction in CKD remains incomplete, but this study highlights the complexity of factors that may be at play, with evidence of both innate immune dysfunction and adaptive immune differences in patients with CKD. Latent CMV infection appears to have an important role in shaping the adaptive immune phenotype and vaccine responses in older adults with and without chronic disease, which raises the possibility that CMV suppression could be a possible therapeutic option to improve adaptive immune responses in this patient population, a concept supported by previous studies in both humans (97) and mouse models (358). The finding of significant hyporesponsiveness to repeat PPV23 in older adults with and without chronic disease in this study suggests a need for re-evaluation of the pneumococcal vaccination strategy in this patient group, particularly in the post-PCV13 era. Only when the nature of immune dysfunction in CKD is clearly defined can we start truly looking for CKD-associated causal mechanisms.

APPENDIX

SONIC study protocol, version 3, July 2016

Supported by wellcome trust

University Hospitals Birmingham NHS
NHS Foundation Trust

University of Birmingham

SONIC study

Investigating the immune **S**ystem in chr**ONIC** kidney disease

Study Protocol

Version 3

July 2016

REC number 15/WM/0057

Clinicaltrials.gov identifier NCT 02535052

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Contents

1. Background & Rationale

1.1 Background

Chronic kidney disease (CKD) affects 8% of the UK population and its prevalence increases exponentially with advancing age. A recent study from primary care identified CKD prevalence of less than 1% of the study population for age groups 18-24 and 25-34, with a dramatic step-wise rise from approximately 3% for age group 45-54 to greater than 40% in individuals aged over 85 years.[3] The management of CKD accounts for >2% of the NHS budget and this is likely to increase significantly with the expansion of the aged population.[4]

CKD severity is classified by the degree of renal impairment (estimated glomerular filtration rate, eGFR) and degree of proteinuria (urinary albumin/creatinine ratio, ACR), as shown in Figure 1.

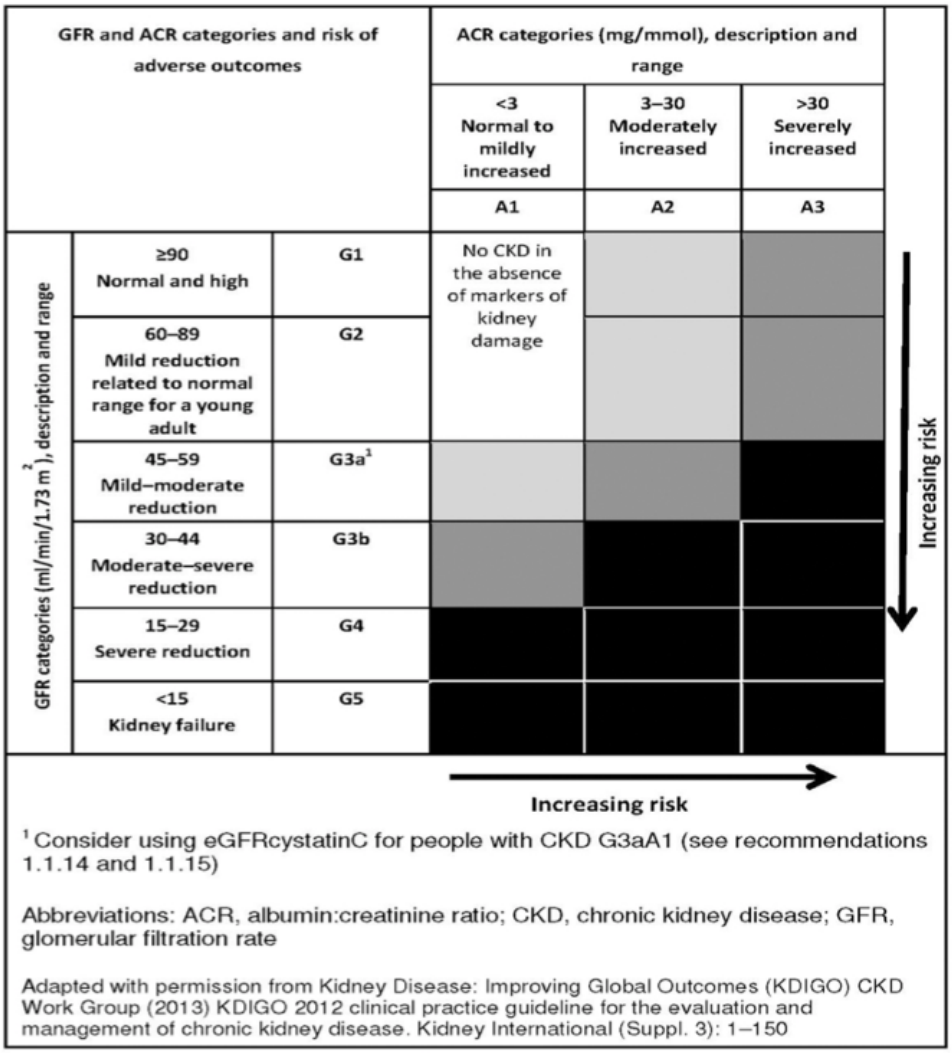


Figure 1. Classification of CKD using eGFR and ACR categories. Sourced from reference [1]

In patients with CKD there is a known diminished immunocompetency, resulting in poorer responses to vaccines, bacteria and viruses [5]. This strongly implies there is a defective adaptive immune system in these patients. The consequence of this is an exponential increase in the risk of infection in patients with CKD, with resultant increased hospitalisation and mortality. One year mortality in patients with end stage renal disease is 13%, with infection accounting for almost 1 in 5 deaths, second only to cardiovascular disease [2]. Mortality related to sepsis is 30-50 times greater in dialysis patients than the general population [6]. Of note, the risk of death increases in a step-wise manner with reduction in eGFR even in individuals with mild/moderate renal impairment and differences in susceptibility to infection are starker in younger age groups [5, 6]. Although mortality from cardiovascular disease (CVD) has markedly reduced over the past 20 years through innovations in diagnosis and patient management, mortality from infection in patients requiring renal replacement therapy remains largely unchanged (Figure 2).

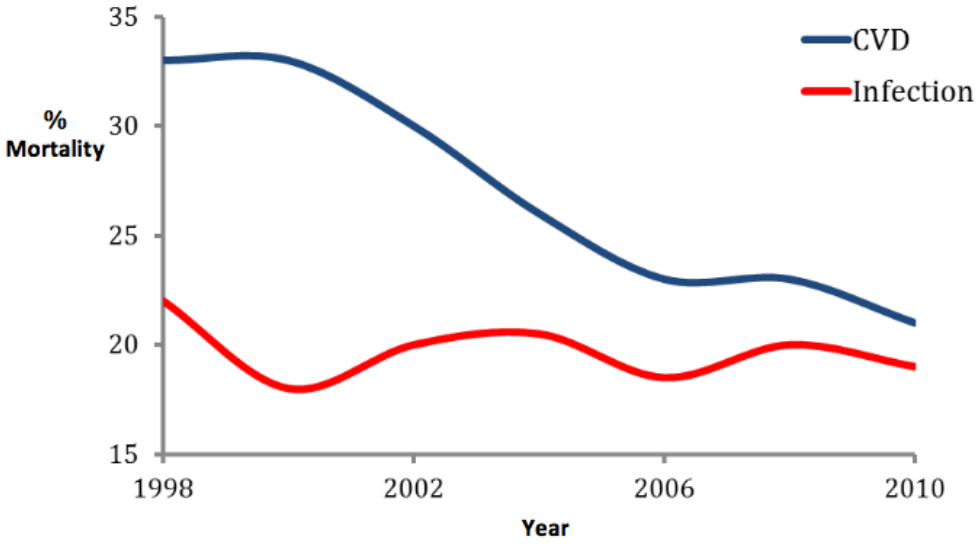


Figure 2. Cause of death in prevalent RRT cohorts by year. Adapted from reference [2].

Public health measures to reduce infection, such as vaccination, are not as effective in CKD patients. Several studies have identified that patients with severe renal impairment exposed to several vaccinations show a lower seroconversion rate, a lower antibody titre peak and a faster decline in protective antibody titres than healthy subjects [7]. This indicates that vaccination is likely to leave CKD patients more susceptible to vaccine-preventable infections than matched individuals without CKD. Furthermore, it suggests that patients with CKD have a defective capacity to mount memory responses and to promote the generation of long-lived antibody responses. The depth of the unequal responses seen in CKD patients is provided in the following examples. Hepatitis B vaccination at the standard schedule recommended for healthy adults has been known for over 20 years to be markedly less effective in dialysis patients, with the rate of early seroconversion in end-stage renal failure (ESRF) patients reported as low as 33%, compared to over 90% seen for healthy adults [7, 8]. In addition there is a marked decline in protective antibody titres in 50% of dialysis

patients at 1 year whereas this is only seen in 15% of healthy adults.[8] An increase in early seroconversion is seen with a double strength dose of hepatitis B vaccine, but this effect is not well sustained over repeated doses.[7] This suggests a defect in the persistence of plasma cells and the maintenance of memory B cell responses to the T-dependent protein vaccine.

Another key element in protecting susceptible individuals from infection is the seasonal inactivated trivalent influenza vaccination. CKD patients can respond well to this vaccine by producing antibody, but nevertheless, a protective antibody level is not achieved in some CKD groups [7] [9]. Lastly, although persistence of protection afforded by pneumococcal polysaccharide vaccination is impacted in patients receiving dialysis, most individuals make a response to this vaccine [7].

Despite these deficiencies several studies have shown a significant reduction in the incidence of pneumonia and overall mortality in dialysis patients who have received pneumococcal [10, 11] and seasonal influenza vaccinations [10, 12]. Collectively, these studies point to a picture where antibody responses to vaccines are commonly induced, but not sustained. Nevertheless, the data also indicate that vaccination can work in this population and thus improving the efficacy of this preventative strategy should be a major aim of future work.

Current national and international guidelines for management of patients with CKD recommend routine immunisation with the seasonal influenza vaccine of all patients with eGFR less than 60ml/min (CKD G3 – 5) and the pneumococcal capsular polysaccharide vaccine (Pneumovax 23) for all individuals over the age of 65 and younger patients with higher risk of pneumococcal disease: those with severe renal impairment (eGFR less than 30ml/min, CKD G4 – 5), nephrotic syndrome, a renal transplant or those undergoing dialysis.[13-15]

1.2 Immune defects associated with renal impairment

Despite the massive clinical impact of CKD-associated immunosuppression on patient life-expectancy and quality of life, the full mechanisms that underlie the devastating relationship between CKD, infection and immune dysfunction are largely unknown. Although some factors, such as uraemia, have been shown to contribute to perturbed immune homeostasis [16], the full range of factors and how they interplay or relate to disease severity have not been identified, largely because immune function in these patients has not been studied in a comprehensive, methodical manner.

To understand the factors causing immunosuppression, we first need to characterise its nature - this remains incompletely understood and the impact of renal impairment alone (isolated from dialysis procedures or age) has not been widely studied. The majority of studies evaluating adaptive immune system function in renal impairment have focused on comparing immune parameters from healthy controls with individuals undergoing haemodialysis. Haemodialysis patients are very much at the extreme end of the spectrum of renal impairment and the process of dialysis itself may be acting as a confounder in these studies, as it can independently alter the immune phenotype through cell activation and removal of key cytokines [17, 18]. Without improved understanding of where the defects lie in the development, maintenance, regulation and implementation of immune functions, potential practical approaches to enhance the efficacy of vaccination and control of infection in CKD patients cannot be identified.

Severe renal impairment and haemodialysis are associated with chronic inflammation as evidenced by elevated serum levels of pro-inflammatory cytokines such as TNF-alpha [19, 20] and a greater

proportion of activated T cells [21]. A chronic pro-inflammatory state has been linked to the greater incidence of atherosclerosis and poorer immune responses seen in this population [20, 22, 23].

Increased T cell turnover and apoptosis has been reported in some studies evaluating dialysis patients. This leads to depletion of naïve and central memory T cells, thus reducing the capacity of the adaptive immune system to recognise new antigens and respond to previously encountered antigens [20, 24]. Reduced B cell numbers and proportion of naïve B cells, together with increased apoptosis are also reported in dialysis patients [25]. The differentiation of B cells in response to antigen challenge into antibody-producing plasma cells in CKD has not been widely studied.

The above T cell features are similar to what is observed in healthy elderly individuals – an "aged" phenotype. Immune response to CMV infection, in particular, the expansion of terminally differentiated memory T cell population (CD4+/CD8+CD28null) has been associated with the "aged" phenotype and accelerated atherosclerosis [26]. This expanded population is also observed in CKD patients [27].

The above observations suggest that there is a dysregulation of immune responses in CKD, rather than an abject failure of immune function: the system as a whole is overactivated (chronic inflammation, expansion of terminally-differentiated T memory cells) at the expense of key specific antigen responses (e.g. poor vaccine response). Our preliminary data supports this dysregulation hypothesis (see Figure 3): CKD individuals from our centre had significantly lower IgG antibody titres to historic antigens (*Haemophillus influenzae* and tetanus toxoid), but significantly higher titres to persistent antigens (Cytomegalovirus, CMV – associated with an ageing phenotype and expansion of terminally-differentiated T memory cells) when compared to healthy age matched controls.

This study will holistically characterise the degree of adaptive immune response dysregulation in a group of patients with mild to moderate renal impairment.

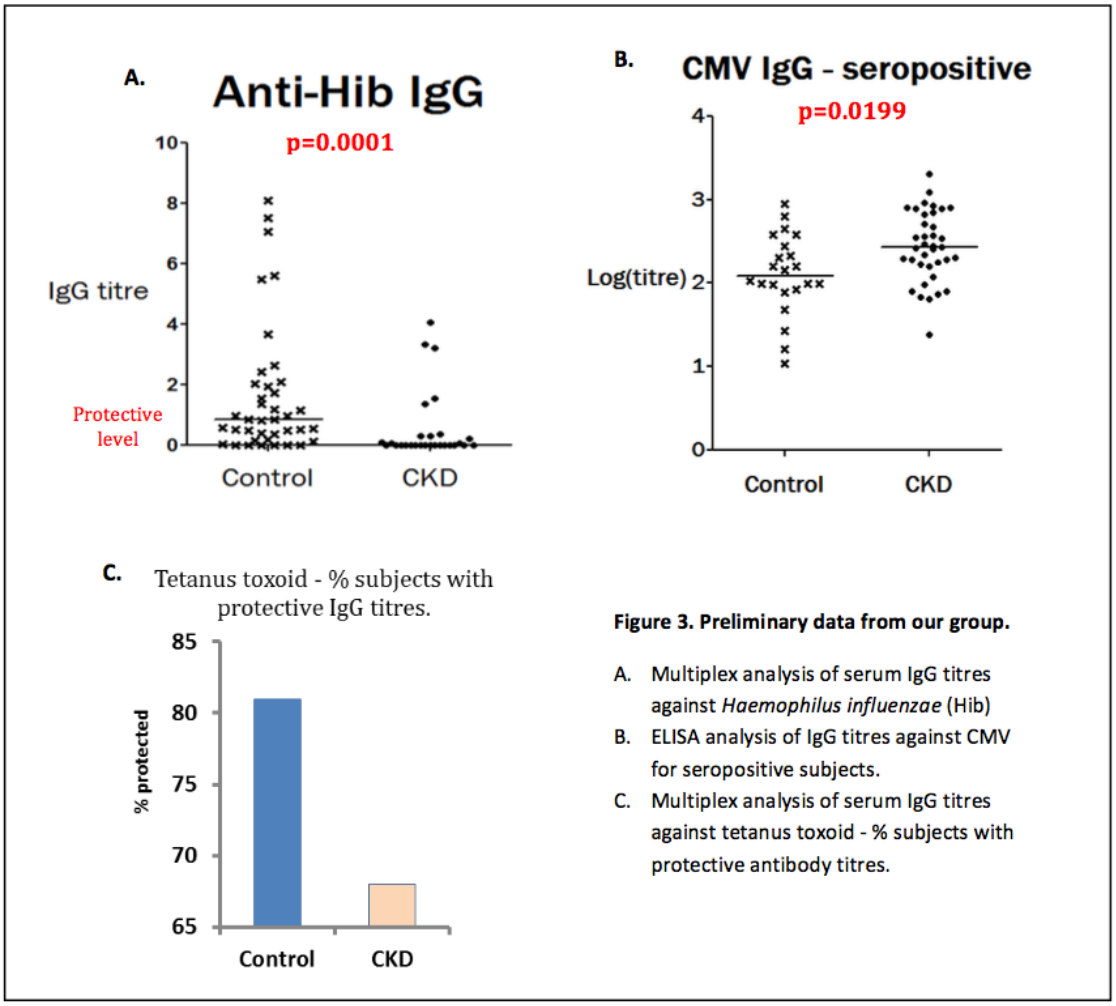


Figure 3. Preliminary data from our group.

A. Multiplex analysis of serum IgG titres against *Haemophilus influenzae* (Hib)
B. ELISA analysis of IgG titres against CMV for seropositive subjects.
C. Multiplex analysis of serum IgG titres against tetanus toxoid - % subjects with protective antibody titres.

1.3 Hypothesis

CKD patients with an eGFR between 15 and 60 ml/min have a dysregulated adaptive immune response to new antigens when compared to healthy age and gender-matched individuals.

These patients are therefore likely to respond less well to an external antigen challenge e.g. the seasonal influenza and pneumococcal polysaccharide vaccines.

1.4 Rationale for this study

A systematic interrogation of multiple components of the adaptive immune system in patients with mild to moderate renal impairment (CKD G3-4) and responses to external antigen challenge by way of vaccination compared to healthy age and gender matched individuals will allow a holistic assessment of the adaptive immune response in this group of patients. Utilising influenza and pneumococcal polysaccharide vaccines will allow real-time assessment of the kinetics and magnitude of T-cell dependent and T-cell independent immune processes respectively.

Without improved understanding of where the defects lie in the development, maintenance, regulation and implementation of adaptive immune functions, potential practical approaches to enhance the efficacy of vaccination and control of infection in CKD patients, which can in turn reduce patient mortality, cannot be identified.

2. Study Objectives

This study has 2 main aims.

Aim 1 (observational):

To investigate differences in the immune systems of patients with mild-moderate CKD and healthy individuals, in particular the relationship between innate and adaptive cell subsets and their function.

Aim 2 (observational with clinically recommended intervention):

To investigate differences in the response of adaptive immune system to external antigen challenge in patients with mild-moderate CKD compared to healthy individuals through real-time assessment of T-cell dependent pathways with seasonal influenza vaccination and T-cell independent pathways with pneumococcal polysaccharide vaccination.

Vaccination in this study will not be assessed for efficacy or tolerability, as the proposed vaccines are already licensed for use in adults for the clinical indications shown in the inclusion criteria. Vaccination will simply be used as a tool to evaluate the difference between immune responses to external antigen challenge in 2 different subject groups, where the exposure of interest is renal impairment (CKD).

Several outcomes will be evaluated:

Primary Outcome

- The proportion (%) of CKD patients having adequate response and protective antibody titres following vaccination with seasonal influenza and pneumococcal polysaccharide vaccination

(as defined in the table below) compared to healthy controls. This will investigate effect of renal failure on vaccine response, not the vaccine efficacy.

Vaccine	Protective antibody titre	Adequate response
Trivalent inactivated influenza	>1:40 (haemagglutinin inhibition assay)[28]	4-fold increase in antibody titre from baseline[28]
Pneumococcal capsular polysaccharide	> 1.3microg/ml (ELISA) [29]	4-fold increase in antibody titre or 2-fold increase in antibody titre if baseline titre >1.3microg/ml [29]

Secondary Outcomes

- Proportion (%) of CKD patients and controls maintaining protective antibody titres up to 6 months following vaccination.

Investigatory Outcomes

- To investigate the relationship between eGFR and proportions of innate and adaptive immune cell subsets and their function at baseline and after antigen challenge (vaccination).
- To investigate the relationship between eGFR and the incidence of infectious disease requiring hospitalisation, GP consultation and/or antibiotics over a period of 6 months.

3. Design

This is a prospective clinical study to investigate the effect of chronic kidney disease on normal physiological processes involved in the immune response to external antigen challenge. Two non-investigational medicinal products (seasonal trivalent influenza and pneumococcal polysaccharide vaccines) will be administered to participants with chronic kidney disease and age and gender matched healthy controls to stimulate the physiological response of the immune system. The immune systems of healthy controls and CKD patients will be phenotyped and compared at baseline. Following antigen challenge (vaccination) the differences in T and B cell phenotype and activation, together with antibody responses will be investigated to understand the effect of CKD on immune system function.

In line with current national vaccination guidelines and the EU licensing for both vaccines, only individuals aged over 65 years will be recruited to the study. We aim to vaccinate a total of 100 study subjects, where 50 will have CKD (eGFR between 15 and 60ml/min) and 50 will have normal renal function (please see section below for sample size calculation). Vaccination as part of this study thus forms part of routine clinical care for both CKD patients and control subjects. We aim to vaccinate an approximately equal number of CKD patients with stages G3 and G4.

The selection of both groups of participants is described in the section below. Baseline immunophenotyping will be performed on all participants prior to vaccination as described in the laboratory investigations below.

4. Statistics

The primary outcome for this study is investigatory. There is little published literature on global immunophenotyping for non-immunosuppressed pre-dialysis CKD patients to inform study sample size calculations for future studies. To our knowledge, this study is novel in its design, incorporating a controlled antigen challenge and quantitative functional analysis of the adaptive immune response in this cohort of patients.

Our previous experience in immunosuppressed CKD patients (vasculitis) has shown a correlation between antibody responses to vaccines and lymphocyte subset numbers/proportions (unpublished data). Similar findings have been shown in some [30-32], but not all studies involving healthy elderly individuals [33, 34]. It follows, therefore, that by ensuring a patient sample size large enough to detect a difference in antibody response to vaccination, we should be able to identify any differences that may exist in the adaptive immune response to vaccination as a whole that may be attributed to eGFR.

Few studies have evaluated influenza and pneumococcal polysaccharide vaccine responses in pre-dialysis CKD patients – the majority of literature pertains to dialysis and transplant patients, and also hepatitis B vaccination, as this remains a large public health concern in haemodialysis units [9, 35-37].

However, we can use studies evaluating vaccination immune responses in healthy elderly individuals to estimate sample size by extrapolating the observed proportional immune response reduction in CKD patients seen in vaccine studies performed in the dialysis population.

The sample size chosen for this study was based on calculations from previous studies evaluating pneumococcal polysaccharide vaccine responses in elderly subjects [38, 39], which were used to estimate the control antibody response, and studies investigating this response in dialysis/CKD patients [40, 41]. Literature on influenza vaccination was felt to be too heterogeneous to be utilised in power calculations due to yearly variations in vaccine components and heterogeneity in study subject selection.

To standardise vaccine response reporting and demonstrate a better measure of central tendency of data, studies frequently report the geometric means of antibody titres for study groups.

From the available literature we expect patients with CKD given Pneumovax to have an approximately 2-fold lower response than controls when pre- and post-vaccination antibody titres are measured on a log scale:

Log(CKD post)-log(CKD pre) = 0.5*(log(control post)-log(control pre))

The following tables summarise the calculations that were performed to estimate the change in antigen-specific IgG in healthy elderly controls, and to calculate the sample size required to detect the above reduction in response in CKD patients with 80% power.

Published results from 2 studies were used in the sample size calculation: 1 – Jackson et al [39], 2 – Lazarus et al [38]. The reported geometric means (together with 95% confidence intervals) before and after pneumococcal polysaccharide vaccination were obtained for each study. These data were log-transformed and used to estimate the standard deviation of the antibody increase following

vaccination. It was assumed that pre- and post-vaccination values correlated with each other and the degree of correlation was estimated using results from study 1. A sample size was then calculated using data from study 2, based on 80% power to detect a 2-fold reduction in antibody titre difference in CKD patients as measured using a logarithmic scale, assuming 2-tailed t tests would be used with 5% significance levels.

Pn serotype	Geomean 2 pre	Lower CI pre	Upper CI pre	Geomean 2 post	Lower CI post	Upper CI post
4	0.13	0.11	0.17	0.7	0.54	0.93
6B	0.4	0.29	0.55	2.58	1.75	3.8
9V	0.31	0.24	0.41	2.72	1.99	3.7
14	0.93	0.64	1.35	9.7	6.33	14.86
18C	0.49	0.36	0.66	6.05	4.19	8.74
19F	0.68	0.52	0.89	3.92	2.94	5.22
23F	0.37	0.28	0.5	2.12	1.5	3

Pn serotype	Post-pre 2 (log)	Estimated correlation	eSD2 with correlation	Required total sample size
4	0.731			
6B	0.810			
9V	0.943	0.390	0.566	48
14	1.018	0.693	0.872	96
18C	1.092	0.689	0.823	74
19F	0.761	0.604	0.673	100
23F	0.758	0.500	0.675	102

As seen in the above table, approximately 50 individuals per group would be required to show the required effect size in vaccination response (total sample size approximately 100).

A feasibility study was performed by the research team to ensure an adequate number of CKD patients could be recruited in the proposed timeframe. All patients attending CKD clinics at UHBFT during the month of August 2014 were screened for eligibility based on electronic clinical records and their vaccination history was confirmed with community services (GP). A total of 405 patients were due to attend CKD clinics, of which 73 (18%) were eligible for the study based on screening clinical records only. Thirty individuals were then excluded based on vaccination history later obtained from community records, leaving 43 eligible individuals (10.6% total). Assuming a 50% uptake rate for participation in the study, we estimate approximately 20 individuals could be recruited per month. The annual influenza vaccination season runs from September to December (4 months) and given the above results, we estimate to be able to recruit approximately 80 individuals per year, thus confidently meeting our target sample size within 2 years.

Differences in baseline clinical characteristics and UHBFT laboratory parameters will be analysed using parametric and non-parametric tests as appropriate. This type of analysis will also be performed for the primary outcomes and majority of secondary and investigational outcomes defined in Section 2.

For exploration of factors associated with poor response to vaccination analysis will be performed using logistic regression. This will initially be unadjusted and then repeated adjusted for baseline covariates.

In all statistical analyses, values of $p < 0.05$ will be considered statistically significant.

5. Selection & Withdrawal of Participants

5.1 Recruitment

Study subjects will be recruited at the University Hospitals Birmingham NHS Foundation Trust (UHBFT) by members of the research team. All study subjects will be given sufficient time to decide whether they would like to take part in the study, during which they will be given both verbal and written information with enough time to read it, time and opportunity to ask questions and receive answers from the research team, and time for their understanding of the study to be checked by the researchers.

5.1.1 CKD patients

Available clinical notes of CKD patients due to attend follow-up clinic at the UHBFT will be screened by the research team for eligibility to study entry. The research team will also obtain vaccination records from individuals' GPs as part of a service improvement project (audit) that will run alongside the study. This initial screening will be undertaken for clinics running during the spring/summer months to identify eligible participants before the seasonal influenza vaccination season begins (usually running from September to December).

Where possible, all provisionally eligible patients attending scheduled clinic follow-up will be informed of the study by letter prior to clinic attendance. This invitation letter will be sent to individuals together with their clinic appointment letter and will be accompanied by a patient information sheet (PIS). The invitation letter will contain contact information for members of the research team, to allow subjects who are interested in participating to contact us directly for further information. Where possible, potential participants will be screened in advance and PIS with covering letter will be sent prior to clinic attendance, but in the case of ad hoc or late booking CKD clinic attendees, patients will be approached by the research team on the day of clinic.

Provisionally eligible patients attending CKD follow-up clinics (in spring/summer months) will be approached by the research team, given both verbal and written information about the study and asked if they would like to participate.

The research team will administer a screening questionnaire to those individuals who are interested in participating in the study to ensure all inclusion criteria and none of the clinical exclusion criteria are met (see below). If eligibility is confirmed and the patient is happy to participate in the study, the research team will ask for written consent to participate in the study and will arrange the first study clinic visit (during vaccination season). Participants will be informed that a letter will be sent to their GP explaining their recruitment to the study, together with the study procedures, to enable primary care records to be updated.

Any patients who are not willing to participate in the study will be given written information about clinically recommended vaccinations for CKD patients. The clinical team will also include these recommendations in their routine clinic letter, so that patients can have their vaccinations in the community.

5.1.2 Control subjects

Control subjects will be age and gender matched with the CKD patient cohort. To maximise recruitment, 2 avenues will be utilised as described below.

5.1.2.1 Patient relatives

Individuals who accompany CKD patients to routine follow-up clinics will be approached by members of the research team and screened for eligibility to participate in the study. They will be given the opportunity to discuss the study in more detail and receive further written information (PIS). Individuals will then be asked if they would like to participate in the study with a plan to receive the two recommended vaccinations at a later date, where possible coinciding with the CKD patient's first study clinic appointment. If eligibility is confirmed and the subject is happy to participate in the study, the research team will ask for written consent. GP vaccination and medical records will be sought by the research team to confirm study eligibility between this initial contact and first study visit (proposed vaccination date). Individuals who, after obtaining vaccination records, are not eligible for study inclusion, will be contacted by the research team both by telephone and post to inform them of this, and thus not included in the study. Participants will be informed that a letter will be sent to their GP when they have been vaccinated as part of the study to enable primary care records to be updated. Any subjects who are not willing or eligible to participate in the study will be given verbal information about relevant clinically recommended vaccinations.

5.1.2.2 Birmingham 1000 Elders Group

Age and gender matched healthy subjects will be sought from the Birmingham 1000 Elders Group, a cohort of healthy subjects aged over 65 years. Inclusion and exclusion criteria, together with anonymised demographic data (age and gender only – to allow matching) for recruited CKD patients will be submitted to the Birmingham 1000 Elders Cohort lead - Prof Janet Lord, and a list of eligible individuals from the cohort will be obtained. . Eligible subjects will be invited to participate in this study by post and a PIS will also be sent. This will then be followed up by the research team with a telephone call to discuss the study in more detail, screen for inclusion and exclusion criteria and ask eligible individuals if they would be happy to attend a study clinic appointment. Subjects will also be asked for verbal consent to obtain their vaccination history and most recent renal function from primary care. Subjects who are happy to attend the study clinic will then be sent a study clinic appointment letter and their vaccination history and recent renal function will be obtained from primary care by the research team. Subjects who are subsequently found not to be eligible for inclusion in the study following primary care data gathering will be informed via post and by telephone by a member of the research team, and thus not recruited to the study.

To maximise recruitment from this cohort, the research team may also approach members of the Birmingham 1000 Elders group at the annual Age Well meetings held by Prof Lord for this cohort. A member of the research team, primarily Dr Nadya Wall, will present a summary of the study to the group, distribute the PIS and allow subjects the opportunity to ask questions about the study. Individuals who are eligible and willing to participate will be asked for written consent to take part in the study, and their vaccination records and renal disease status (including renal function) will be checked with primary care before the first scheduled study visit. Subjects who are subsequently found not to be eligible for inclusion in the study following primary care data gathering will be informed via post and by telephone by a member of the research team, and thus not included in the study.

Subjects attending the study clinic will be screened for eligibility by members of the research team using a questionnaire, further verbal information will be given and further written information will be offered. If eligibility is confirmed and the individual is happy to participate in the study, the research team will ask for written consent to participate in the study and will administer vaccinations and take blood and urine samples at this first clinic visit.

Participants will be informed that a letter will be sent to their GP explaining their recruitment to the study, together with the study procedures, to enable primary care records to be updated. Any subjects who choose not to participate in or are found not to be eligible for inclusion into the study will be given verbal information about clinically recommended vaccinations.

5.1.2.3 Subjects without historical renal function results

Individuals with no known renal disease and no historical renal function records will be included in the study (as study vaccines will form part of routine care for these subjects), but their disease grouping will be determined by the first study eGFR. If such subjects are found to have an eGFR in CKD disease range, they will be informed both by letter and telephone call and the research team will ask permission to inform their GP for further review and investigation as necessary. Individuals without historical renal function recruited as controls, who are subsequently identified to have CKD on their first study blood sample, will then be transferred to the CKD arm of the study.

5.2 Inclusion Criteria

- Subjects of any gender aged 65 or over will be included in the study.

5.3 Exclusion Criteria

- **Clinical:**
    - Subjects aged under 65 years of age
    - Subjects who do not have capacity to consent to study participation as defined by the Mental Capacity Act 2005
    - Subjects with comorbidities that are associated with an immunosuppressed state including, but not limited to, the following:
        - Malignancy diagnosed within last 5 years except non-melanoma skin cancer
        - Solid organ (including kidney) or bone marrow transplant recipients
        - Blood borne viral infections: HIV, hepatitis B and C
        - Autoimmune disease e.g. vasculitis, rheumatoid arthritis
        - Previous splenectomy or asplenia from any other cause
    - Subjects currently taking any of the following immunosuppressive medications:
        - Systemic corticosteroids e.g. prednisolone/dexamethasone
        - Chemotherapy agents e.g. cyclophosphamide, methotrexate, azathioprine
        - Biological therapies e.g. rituximab, infliximab, etanercept – including any administration within preceding 12 months
    - Subjects who have previously had a serious reaction to influenza vaccination, including anaphylaxis and Guillain-Barre Syndrome within 6 weeks of previous influenza vaccination
    - Subjects who have a severe egg allergy (contraindication to influenza vaccination)
    - Subjects who have previously had a serious reaction to the whole or any components of pneumococcal vaccination

  - Subjects who have an active infection and/or are febrile on assessment
  - For control subjects:
    - eGFR <60ml/min
    - any known renal disease
  - For CKD subjects:
    - eGFR ≤15ml/min or ≥60ml/min
- **Vaccination history:**
  - Subjects who have already received the seasonal influenza vaccine during current year's vaccination season
  - Subjects who have received Pneumovax23© within last 5 years

5.4 Subject Withdrawal

Subjects who wish to withdraw from the study will have no further samples collected, but previously collected samples will be retained within the study unless the participant requests their destruction. Study subjects who do not attend follow-up clinic appointments will be invited to attend again via letter and telephone call. If they do not attend 2 rescheduled appointments, they will be withdrawn from the study by the research team and classed as 'lost to follow-up'. If this occurs within 2 months of recruitment to the study, the research team will aim to recruit another study subject in their place.

Subjects who develop one of the immunosuppressing conditions in the exclusion criteria or are commenced on any immunosuppressant medications during the course of the study will be withdrawn.

5.5 Study Flowcharts

Study subjects will have a maximum of 5 interactions with the research team. The first of these will primarily be face-to-face information-giving and an opportunity for subjects to ask questions. It is intended that this interaction will occur during a routine CKD clinic visit for CKD patients. It is also intended that, as much as possible, the final follow-up visit (6 months) will coincide with a routine CKD clinic appointment. The following flowcharts (Figures 4 and 5) represent the planned flow of subjects through the study process.

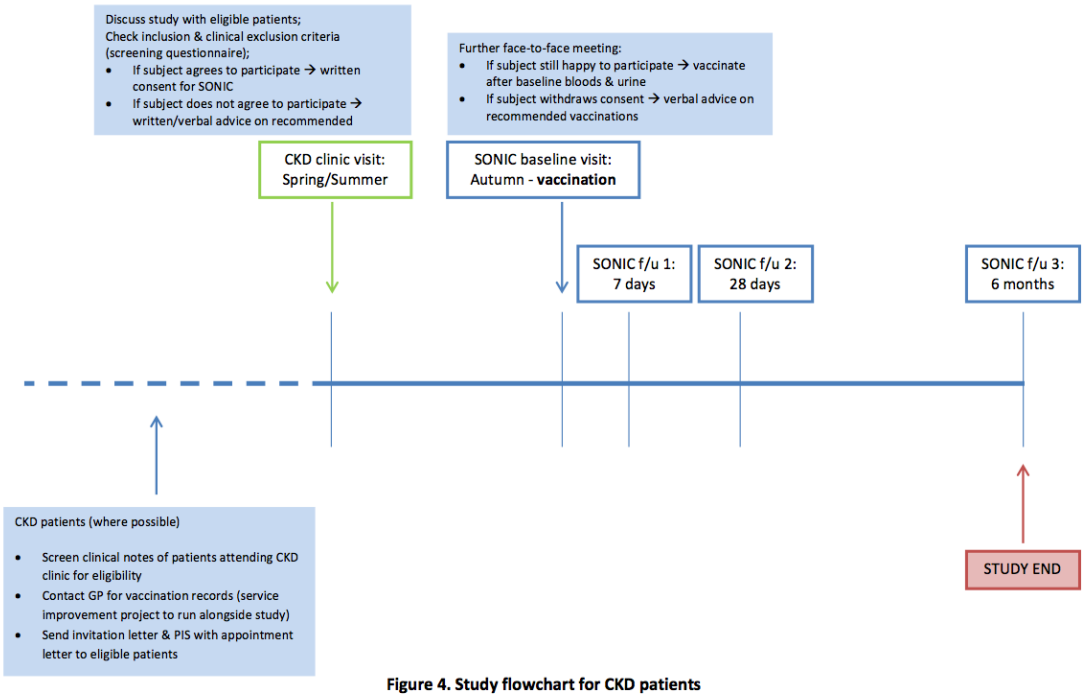


Figure 4. Study flowchart for CKD patients

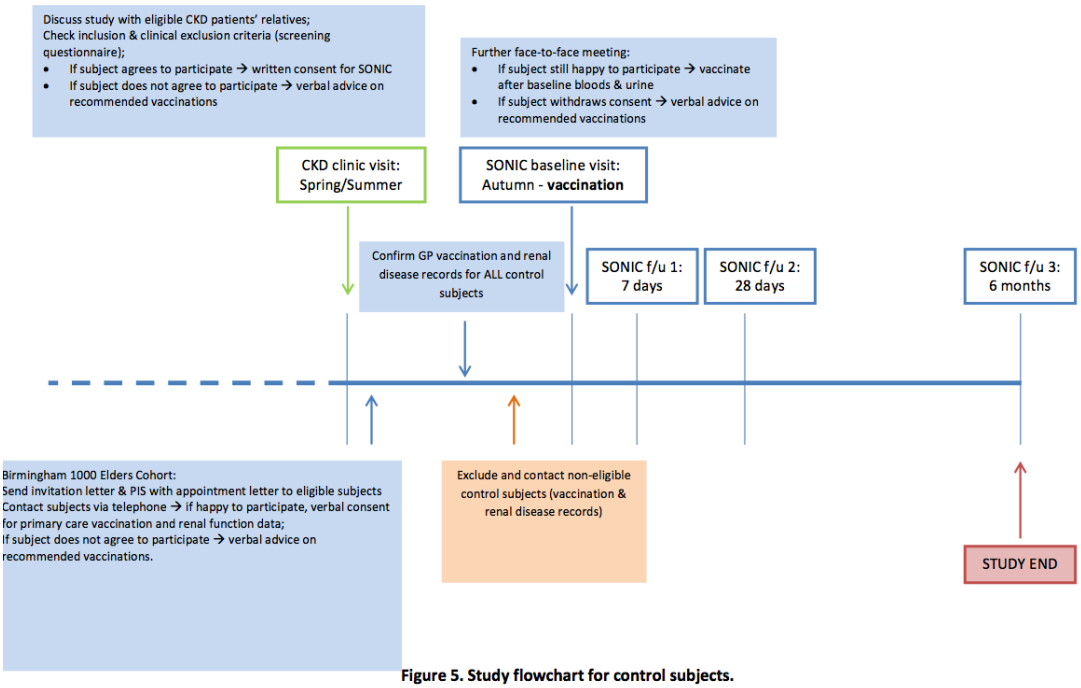


Figure 5. Study flowchart for control subjects.

The following table describes the proposed interventions and monitoring which will be undertaken at each study visit.

	Pre-vaccination (baseline)	7 days post-vaccination	28 days post-vaccination	6 months post-vaccination
Patient data				
Demographics	X			
BMI	X			
Medical history	X	X	X	X
Medication history	X	X	X	X
Vaccination history	X			
Inclusion/exclusion criteria	X			
Informed consent	X			
Incidence of infection		X	X	X
Antibiotics received		X	X	X
Adverse events		X	X	X
Patient survival		X	X	X
Haematology				
Full Blood Count with differential white cell count	X	X	X	X
ESR	X	X	X	X
Biochemistry				
Renal profile with eGFR	X	X	X	X
Alkaline phosphatase	X			
Albumin	X			
Calcium	X			
Phosphate	X			
Vitamin D	X			
hsCRP	X	X	X	X
Lymphocyte function				
Whole Blood	X	X	X	X
Serum	X	X	X	X
Urine				
Albumin/creatinine ratio	X	X	X	X
Drug administration*				
Seasonal inactivated trivalent influenza vaccine	X			
Pneumococcal capsular polysaccharide vaccine (Pneumovax 23©)	X			

* Vaccines administered **after** patient data and sample collection at baseline

6. Study Challenge Agent

Two non-investigational medicinal products will be used in this study: the seasonal inactivated trivalent influenza vaccination and the pneumococcal capsular polysaccharide vaccine – Pneumovax 23©, Merck Pharmaceuticals, US. Both vaccines have appropriate marketing authorisation and license for use in the UK for people aged 65 years and over.

The current WHO recommendations for the composition of the seasonal inactivated trivalent influenza vaccination for the 2014/2015 Northern Hemisphere influenza season are:

- an A/California/7/2009 (H1N1)pdm09-like virus;
- an A/Texas/50/2012 (H3N2)-like virus;
- a B/Massachusetts/2/2012-like virus.

The vaccines will be dispensed by the UHBFT pharmacy, labelled according to regulations and the manufacturer of the influenza vaccine will be determined closer to the vaccination season, as this can change between seasons. The influenza vaccine will be administered intramuscularly by appropriately trained members of the research team, at the recommended single dose determined by the manufacturer.

The Pneumovax 23© vaccine contains capsular polysaccharides for 23 *Streptococcus pneumoniae* serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. This vaccine will also be administered intramuscularly by appropriately trained members of the research team at a single 0.5ml dose, as recommended by the manufacturer.

All subjects recruited to the study will be expressly asked not to receive the seasonal influenza and pneumococcal polysaccharide vaccines in primary care prior to the study vaccination visit to ensure they do not receive a double dose of vaccine. This will also be checked by a member of the research team on the day of study vaccination to ensure subject safety. This information will be included in the study PIS and GP letter of study participation.

6.1 Drug Accountability

All members of the research team are responsible for ensuring that all study drugs at the site are inventoried and accounted for throughout the study. A clinical study stock of both vaccines will be provided by UHBFT pharmacy to the Principal Investigator and stored at the NIHR/Wellcome Trust Clinical Research Facility at UHBFT in a limited access area or in a locked cabinet under appropriate environmental conditions. Study drugs will be handled in strict accordance with the study Protocol and accountability will be maintained by the research team – dispensing and prescription information will be recorded for each individual study subject and filed in ante-chronological order. These records, together with any unused/returned study drugs (if applicable) will be available for verification by the Sponsors' site monitor. The destruction of any unused study drugs (expired/unexpired) or used returned study drugs will be recorded on a drug return/destruction form. Destruction of study drugs will occur as per local pharmacy policy. Study drugs will be supplied only to subjects participating in the study. However, individuals who fulfil inclusion criteria and request vaccination in the clinic setting, but do not wish to participate in the study or fulfil exclusion criteria, will be offered vaccinations as part of routine clinical care provision and these drugs will be provided by the UHBFT pharmacy outside of the remits of the study.

6.2 Concomitant Medication

Very few drug interactions are reported for the study drugs. Merck Pharmaceuticals report a reduced immune response to ZOSTAVAX® vaccination (a live attenuated herpes zoster virus vaccine, used for prevention of shingles in individuals aged over 50 years) when given concurrently with Pneumovax 23©. It is advised that administration of these vaccines are separated by a minimum of 4 weeks.

The manufacturer information for the seasonal inactivated trivalent influenza vaccine will be consulted for reported drug interactions, when it is available.

All concomitant medications, including insulin, inhalers and medicated creams will be recorded by the research team at baseline for every study subject to identify potential confounders in later analysis. This information will be reviewed and changes recorded at every follow-up visit.

6.3 Adverse Events

The following adverse events are reported for both study drugs in the approximate frequency as indicated in the table below.

Adverse reaction	Pneumococcal capsular polysaccharide vaccine reported incidence	Influenza vaccine reported incidence
Local reactions		
Pain/soreness/tenderness	Very common	Very common
Swelling/induration	Rare	Common
Erythema	Rare	Common
Cellulitis-like reactions at injection site	Rare	Rare
Systemic reactions		
Fever	Common	Very common
Headache	Very common	Very common
Fatigue	Very common	Very common
Myalgia	Very common	Very common
Oculo-respiratory syndrome	Not reported	Very rare
Immune system disorders		
Anaphylactoid reactions	Very rare	Very rare
Urticarial rash	Rare	Rare
Nervous system disorders		
Guillain-Barre syndrome	Not reported	Very rare
Gastrointestinal disorders		
Nausea/vomiting	Common	Common
Diarrhoea	Common	Unknown
Abdominal pain	Unknown	Common
Very common denotes incidence ≥1:10; common denotes incidence ≥1:100 to <1/10; uncommon denotes incidence ≥1:1000 to <1/100; rare denotes incidence ≥1:10,000 to <1:1000; very rare denotes incidence <1/10,000; unknown – cannot be estimated from available epidemiological data. Compiled from manufacturer prescribing information and WHO recommendations for above vaccinations.[42-44]		

Serious adverse events are defined by the NHS Health Research Authority (HRA) as an untoward occurrence that:

- Results in death
- Is life-threatening

- Requires hospitalisation / prolongation of existing hospital stay
- Results in persistent or significant disability / incapacity
- Consists of a congenital anomaly or birth defect
- Is otherwise considered medically significant by the investigator.

All study subjects will be screened for adverse events following vaccination at follow-up visits by the research team.

Related (felt to have resulted from administration of vaccinations) and/or unexpected events (those not listed in the above table) will be reported to the main Research Ethics Committee by the Chief Investigator within 15 days of them becoming aware of the event, in line with HRA recommendations. All severe adverse events will also be reported to the MHRA using the Yellow Card Scheme by a nominated member of the research team.

7. Study Procedures

7.1 By Visit

All study subjects will have a maximum of 5 interactions with the research team. The first of these will be to receive verbal and written information face-to-face – for CKD patients and their relatives, this will coincide with a routine CKD clinic appointment and therefore not be an additional hospital visit. For healthy elderly control subjects, this initial information giving interaction will be conducted via telephone, during which a further face-to-face meeting will be arranged for individuals interested in participating in the study. A total of 4 study clinic appointments will be held at UHBFT. During the first study visit (baseline) subjects will be recruited to the study – for CKD patients this will coincide with a routine CKD clinic appointment, but for healthy controls this will be a separate visit to UHBFT solely for the purpose of the study. After written informed consent has been obtained, baseline clinical assessments, blood and urine samples will be taken and all subjects will receive both the seasonal influenza and pneumococcal vaccinations. The remaining 3 follow-up visits will be scheduled at 7 days, 28 days and 6 months following vaccination. At each follow-up visit the research team will perform clinical assessments and further blood samples will be taken. Subjects will have completed the study when the 6 month follow-up visit has been completed.

This schedule is shown in the Study Flowchart (Figure 4).

7.2 Laboratory Procedures

Blood and urine samples will be obtained from study participants at every study clinic visit. The UBHFT clinical laboratory will process some of these samples for the following investigations, commonly used in clinical care:

- Urine albumin/creatinine ratio*
- Full blood count with differential white cell count*
- ESR*
- Renal profile with calculated eGFR*
- Serum Alkaline phosphatase
- Serum Albumin

- Serum Calcium
- Serum Phosphate
- Parathyroid hormone
- Serum Vitamin D
- Highly sensitive C-reactive protein (hsCRP)*
- HbA1c – glycated haemoglobin
- Ferritin, transferrin saturation

Items denoted by * will be processed at every visit, with the remainder only at the baseline assessment.

Blood samples drawn from study subjects from every clinic visit will also be processed using research techniques at the University of Birmingham Centre for Translational Inflammation Research and Institute of Biomedical Research. These will be labelled only with anonymised study identifiers. Research techniques will include (but are not limited to) the following:

1. Cell-based assays (whole blood): lymphocyte subset and intracellular cytokine analysis by flow-cytometry; lymphocyte proliferation assays; ELISPOT assays.
2. Antibody-based assays (serum only): quantification of serum antibody levels using ELISA and multiplex methods, together with functional antibody analyses.
3. Complement functional assays (serum only): bactericidal killing assays with substitution of human antibody with mouse sera.

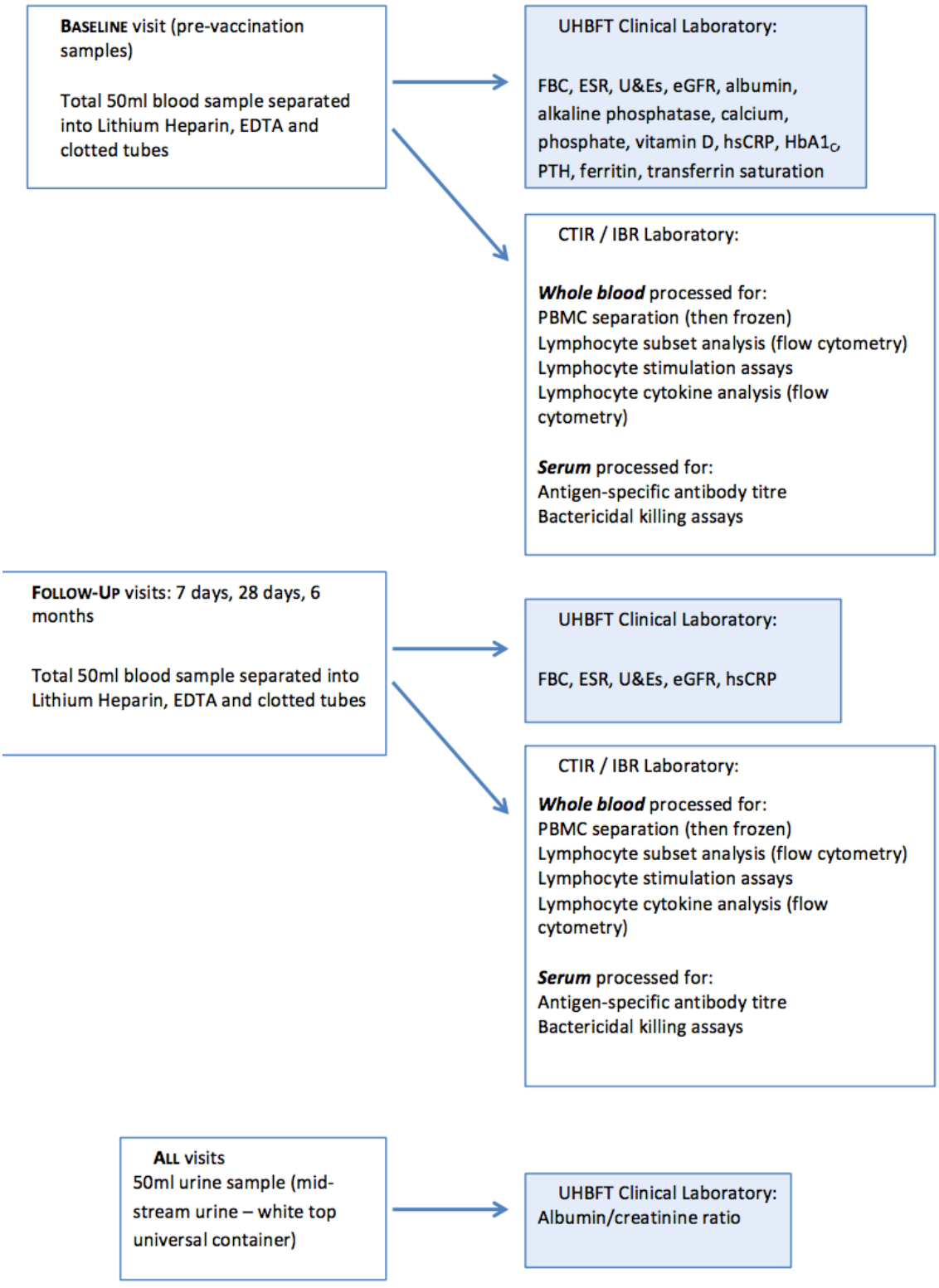
Figure 5 summarises the flow of study subject samples at every study clinic visit.

Any grossly abnormal routine clinical results from any participant (processed by UHBFT clinical laboratory, not research techniques) will be discussed with the individual by members of the research team and they will be advised to seek further medical review e.g. from their GP. No information on results from the research techniques will be discussed with individual study participants.

7.3 Sample Storage

The named Principal Investigator will act as custodian of study subject samples. After collection, study research samples will be anonymised (labelled only with unique study identifiers) and stored by the research team at appropriate conditions (e.g. -80°C freezers/liquid nitrogen) in limited access laboratory facilities (requiring authorised swipe-card) at the University of Birmingham. Only named Investigators will be allowed access to study samples. Samples will be retained until study analysis is complete (intended to be a maximum of 1 year after study end) and will then be destroyed in accordance with University of Birmingham policies.

Figure 5. Sample flow diagram



8. Source Data and Documents

8.1 Data Handling, Record Keeping and Storage

The named Chief Investigator will act as custodian for the study data. The following conditions will be strictly adhered to:

1. All study subject data will be link anonymised:
- Each participant will be assigned a unique numerical study code
- Personal information will be stored separately to anonymised data
- A secure 'link database' will be created to enable matching of study codes to personal information for study analysis and patient identification in the event of abnormal results or SAEs – this will be stored exclusively on NHS servers at the UHBFT.

2. All data will be stored securely:
- The electronic 'link database' will be stored exclusively on secure NHS servers at the UHBFT.
- Personal data in paper format will comprise only the participant's name on the study consent form, and this will be stored in a locked cabinet in a limited access area at the UHBFT to which only named members of the research team will have access.
- Anonymised data in paper format (including medical assessment data collection forms, which will only include the study identifier) will be stored in a locked cabinet in a limited access area at the University of Birmingham. Anonymised data in electronic format will be stored only on password-protected computers on the University of Birmingham network (to facilitate data analysis).
- No anonymised or personal data will be stored on personal computers or external hard drives.
- Analysed data will be stored electronically as described above in anonymised datasets (study numbers removed) in password-protected format accessible only to named Investigators.

3. No data (anonymised/personal information) will be shared with any other study or organisation without further written consent from study participants.

Study subject identifiable data will be securely stored for the duration of the study period and a period of time thereafter to allow analysis to take place. This is intended to be no longer than 12 months for personal data applicable to the study and 5 years for data generated during the study (anonymised), after which both paper and electronic data will be destroyed in accordance with UHBFT regulations. Clinical data on hospital data systems for individual study participants will be stored according to the UHBFT data management policy, as this data may be used by clinicians in the future to inform patient care.

Procedures that will be followed for the storage of study samples (urine and blood) are described in Section 7.3.

8.2 Data Access and Quality Assurance

The named Investigators will allow study-related monitoring, audits, Research Ethics Committee review and regulatory inspectors (where appropriate) by providing direct access to study source data and associated documents e.g. consent forms, UHBFT laboratory results etc.

9. Ethics & Regulatory Issues

9.1 Ethics Approval

This protocol and related documents have been submitted for ethical approval from the Local Research Ethics Committee via an application to the Integrated Research Application System (IRAS). The study will not commence until ethical approval has been granted.

This study will be conducted in compliance with the principles of Good Clinical Practice and the Declaration of Helsinki (1996).

9.2 Patient Consent

Study participants will be identified at the UHBFT as described in Section 6. The research team will seek to obtain written consent for study participation, with subject signature on a written Consent Form (see Appendix).

A full Patient Information Sheet (PIS) will be provided to every potential participant prior to recruitment (by post with invitation letter to participate in study and clinic appointment letter) and the risks and benefits of study participation will be clearly explained by members of the research team prior to recruitment. Subjects will be given ample time to read written information provided and the opportunity to ask any questions of the research team. Queries and concerns from subjects will be answered to the satisfaction of the potential participant.

Sufficient time will be given to individuals to decide whether or not to participate in the study. It will be made expressly clear to individuals via written and verbal information that patients are under no obligation to take part in the study and they have the right to withdraw from the study, without giving a reason, at any time. If the individual agrees to participate in the study they will be asked to sign and date the Consent form, which will be countersigned by a member of the research team. The study subject will be provided with a copy of the Consent form and copies will be filed in hospital clinical notes and the Study File. Study subjects will be offered the opportunity to ask questions about the study throughout its duration. New information that may be relevant to subjects' willingness to continue taking part in the study will be shared with them in a timely manner by the research team.

9.3 Confidentiality

All personal information collected and recorded on all document forms during the study (including electronic) will be regarded as strictly confidential and will be handled and stored in accordance with the 1998 Data Protection Act.

9.4 Indemnity

The Sponsor - University of Birmingham, has in force a Public Liability Policy and/or Clinical Trials policy providing cover for claims for negligent harm suffered by study participants arising from the management and design of research projects and the activities of this study are included within that coverage.

The NHS indemnity scheme or professional indemnity held by members of the research team will apply to meet the potential legal liability of investigators that may arise from harm to participants in the conduct of the research.

10. Publication Policy

The research team intend to report and disseminate the results of this study at international conferences and peer-reviewed scientific journals. A lay summary of study results will also be published as part of a newsletter for the local CKD patient group – Queen Elizabeth Hospital Kidney Patients Association (QEHKPA) and will be presented at the annual Age Well meeting run by Prof Lord for the Birmingham 1000 Elders cohort.

11. Study Steering Committee (SSC)

The SSC will assess study progress, incidence of SAEs and other aspects of the study including recruitment. The SSC will meet at 3-6 monthly intervals.

12. Data Monitoring Committee (DMC)

The DMC will also meet at 6 monthly intervals.

13. Financial Arrangements

This study has received funding from the Wellcome Trust as part of a Joint Basic and Clinical PhD Fellowship (Reference Number: 105479/Z/14/Z).

14. Signatures

Dr Nadezhda Wall

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16 Appendix

Baseline data collection tool

Follow-up data collection tool

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BASELINE MEDICAL ASSESSMENT

Investigating **SecONdary Immunodeficiency in Chronic kidney disease - the SONIC study.**

Study identifier	**Date**	**Name & Signature of Investigator**

Renal diagnosis	
Medical History	IHD PVD CVA/TIA DM (Type 1 / Type 2) HTN
Vaccination history Born in UK? Y / N Childhood immunisations? Y / N Tetanus up-to-date? Y / N	Last influenza vaccine: Last pneumococcal polysaccharide vaccine:
Current medications	**Temperature today:** _____
Current smoker? Y / N	_____ / day, _____ years
Former smoker? Y / N	_____ / day, _____ years, quit date _____
Current alcohol consumption Y / N	_____ units / week

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FOLLOW-UP MEDICAL ASSESSMENT

Investigating **Sec**ONdary **I**mmunodeficiency in **C**hronic kidney disease - the **SONIC** study.

Study identifier	**Date**	**Timepoint** 7 days / 28 days / 6 months	**Name of Investigator**

New Medical Events since last visit	Hospital admission? Y / N Details Infections requiring GP visit/antibiotics/time off work? Y / N Details Cardiovascular events? Y / N Details Other new diagnoses? Y / N
Current medications	

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